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I. Extracellular metabolites from coprinus macrorhizus microsporus. Isolation and characterization of hydroxylagopodin B. II. The cell wall composition and structure of coprinus macrorhizus microsporus

Carey Bernard Bottom

Department: Chemistry

I. EXTRACELLULAR METABOLITES FROM COPRINUS MACRORHIZUS MICROSPORUS. ISOLATION AND CHARACTERIZATION OF HYDROXYLAGOPODIN B

II. THE CELL WALL COMPOSITION AND STRUCTURE OF COPRINUS MACRORHIZUS MICROSPORUS

BY

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ABSTRACT

A purple pigment excreted into the culture medium of a mutant strain of the basidiomycete, Coprinus macrorhizus var. microsporus (ATCC 34960) has been isolated and characterized as hydroxylagopodin B based on ultraviolet, infrared, and mass spectral data. A wild-type strain of C. macrorhizus microsporus does not produce the purple pigment, however it does produce lagopodin B which is yellow in solution. Identification of lagopodin B was based on its melting point, the purple color of its alkaline solution, the mass spectrum, and mobility equivalence with a standard by thin-layer chromatography. The production of hydroxylagopodin B in the mutant strain is concomitant with a significant increase in the phenoloxidase activity of the culture medium. Some phenoloxidases possess activity which hydroxylates phenols or their derivatives. Since the structures of hydroxylagopodin B and lagopodin B differed by the substitution of a hydroxy group on the benzoquinone nucleus, it was proposed that hydroxylagopodin was a product of non-specific hydroxylation of lagopodin B as the corresponding quinol catalyzed by the mutant's phenoloxidase. Evidence indicated that the purified phenoloxidase did not hydroxylate lagopodin B under the given conditions, however it is probable that an indirect relationship exists between the production of hydroxylagopodin B and the increase in phenoloxidase activity.

The cell-wall structure of wild-type C. macrorhizus microsporus was also studied.

A unique, alkali-soluble polysaccharide has been isolated from the cell walls of the basidiomycete, Coprinus macrorhizus microsporus. The polysaccharide, which is primarily glucan in nature, contains a large proportion of α-(1→4)-linked D-glucose residues and a smaller amount of β-(1→3),(1→6) linkages as suggested by methylation, partial acid hydrolysis, periodate, and enzymic studies. Hydrolysis of the methylated polysaccharide gave equimolar amounts of 2,4-di- and 2,3-di-O-methyl-D-glucose; no 2,6-di-O-methyl-D-glucose was identified indicating the absence of branch points joined through O-1, O-3 and O-4. The isolation and identification of 2-O-α-glucopyranosyl erythritol from the periodate oxidation of the polysaccharide suggests that segments of the α-(1→4)-linked D-glucose residues are joined by single (1→3)-linkages. An extracellular enzyme preparation from Sporotrichum dimorphosporum (QM 806) containing both β-(1→3) and α-(1→4) glucanohydrolase activity released 76% of the reducing groups from the polysaccharide. The polysaccharide also contains minor amounts of xylose, mannose, glucosamine and amino acids.

The alkali-insoluble (R-) fraction from the cell walls of Coprinus macrorhizus var. microsporus is a highly-branched glucan, containing α-(1→4), β-(1→3) and β-(1→6) linkages as shown by methylation, partial acid hydrolysis and enzymic hydrolysis. The α-(1→4)-linked segments are joined by occasional β-(1→3) links as suggested by the identification of 2-O-α-glucopyranosyl erythritol in the hydrolysate of the reduced, periodate-oxidized glucan. Hydrolysis of the permethylated glucan gave nearly equimolar amounts of 2,4-di- and 2,3-di-O-methyl-D-glucose. Methylation analysis of the residue from enzymic hydrolysis,

the CORE-fraction, indicated the presence of glucose residues in this fraction also,linked through positions 0-1, 0-3, 0-4 and 0-6. Hydrolysates of the R-fraction also contained mannose, glucosamine and amino acids in addition to glucose.

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to his advisor, Dr. Donald J. Siehr, Professor of Chemistry, for his professional advice and his encouragement during the course of this work. His stimulating discussions provided a valuable addition to the development of my scientific career. Appreciation is extended to other members of the Chemistry Department, Drs. Samir Hanna, William Carroll, Wilbur Tappmeyer, D. Vincent Roach, and Louis Biolsi and to members of the Life Science Department, Drs. Nord Gale and James Hufham. The author also wishes to thank Dr. Milton Feather, Professor of Biochemistry, University of Missouri-Columbia for his participation as a member of the doctoral committee and his critical review of the dissertation. The advice and assistance of his fellow graduate students, especially Gary Magruder, is gratefully acknowledged.

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PART I

Extracellular Metabolites from *Coprinus macrorhizus microsporus*. Isolation and Characterization of Hydroxylagopodin B

Hydroxylagopodin B, a Sesquiterpenoid Quinone from a Mutant Strain of *Coprinus macrorhizus* var. *microsporus*

CAREY B. BOTTOM and DONALD J. SIEHR
Department of Chemistry, University of Missouri
Rolla, MO 65401 U.S.A.

Key Word Index-*Coprinus macrorhizus* var. *microsporus*; Basidiomycetes; mushroom; isolation; characterization; sesquiterpenoid quinone; hydroxylagopodin B.

Plant. *Coprinus macrorhizus* var. *microsporus*. *Source*. Dr. Philip G. Miles, Biology Department, SUNY at Buffalo.

Present Work. A single gene mutant of the basidiomycete *Coprinus macrorhizus* var. *microsporus* (5074) excretes into the liquid culture medium an intense violet pigment which represents a series of possibly related compounds [1]. One of these has now been characterized as hydroxylagopodin B (1) which is clearly related to lagopodin B (2) [2], isolated from *Coprinus lagopus*.

The pigments were isolated by EtOAc extraction of the acidified filtered spent medium. The extract was evaporated *in vacuo* to dryness, the tarry residue redissolved in EtOAc and the pigments extracted into 0.1 M $NaHCO_3$. The bicarbonate phase was acidified

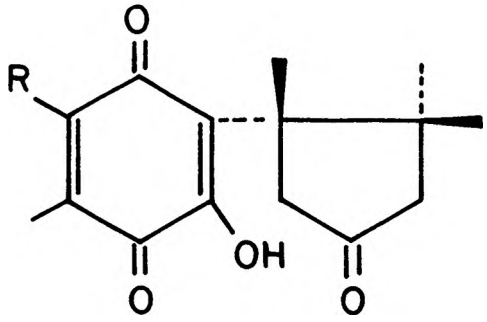
(pH ~ 3) and extracted with C_6H_6. Following evaporation of this extract to near dryness, the residue was dissolved in 2% MeOH-$CHCl_3$ and separated on silica gel; (1) was eluted with 2% MeOH-$CHCl_3$ as the second purple band which yielded red-orange rhombic platelets (from C_6H_6-heptane), m.p. 184-186°. Yield: 3.5 mg./l. of culture medium. Alkaline aqueous solutions of (1) were violet. Its solutions are decolorized by $NaBH_4$ and the color restored on aeration after the addition of $NaHCO_3$. UV λ_{max} (EtOH) 212, 307 and 351 nm. is consistent with a 2,5 dihydroxy-3-6-dialkylbenzoquinone, e.g. helicobasidin [3].

The IR spectrum (KBr) showed OH at 3500 and 3385 cm^{-1}, characteristic (hydroxyquinone) CO absorptions at 1650 and 1627 cm^{-1}, and alkane C-H stretching bands in the region of 2900-3000 cm^{-1}. The cyclopentane carbonyl band, which should appear at ~1750 cm^{-1}, was not present. This suggests that crystalline hydroxylagopodin B exists as a hemiketal structure (3) [3].

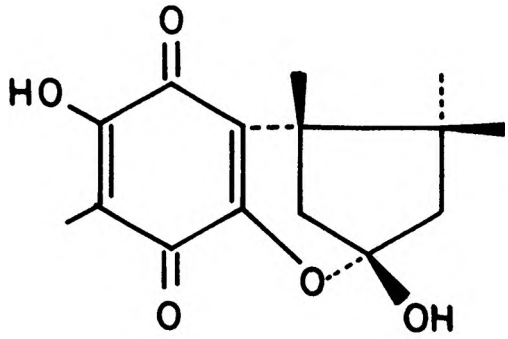
Hydroxylagopodin B has a molecular formula $C_{15}H_{18}O_5$ (M^+ 278), as determined by high resolution MS. Major ions in MS: m/e 280 (5.7), 278 (56.5), 221 (78.3), 194 (100), 166 (23.6). The strong M + 2 peak is observed which is typical of p-benzoquinones [3]. The fragmentation pattern confirms that hydroxylagopodin B is related to lagopodin B, the most abundant ion (100%) in the latter being at m/e 178. The ion m/e 166 arises from the expected elimination of an acetylene fragment from the benzoquinone ring.

Biological significance. The lagopodins have some antibiotic activity. No test of the antibiotic properties of hydroxylagopodin B was made.

Acknowledgements-We thank Dr. Miles for drawing our attention to this organism and supplying cultures. We also thank Mr. R. H. Rice, Department of Agricultural Chemistry, University of Missouri, Columbia for carrying out the mass spectrometry.



(I) R=OH (II) R=H



(III)

Figure 1. Structures of Hydroxylagopodin B (I), Lagopodin B (II), and Hydroxylagopodin B as an Alternative Hemiketal (III)

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APPENDIX A

METABOLITES FROM COPRINUS MACRORHIZUS MICROSPORUS

I. INTRODUCTION

A. GENERAL

Many compounds are produced by microorganisms which do not appear to be essential for growth and usually their production is not associated with the growth phase. These compounds are known as secondary metabolites. In many cases, a specific secondary metabolite is produced by only one species of a genus, or in some cases by only a specific strain. Certain mutations produce a specific metabolite in high yield. In the fermentation industry, especially the production of pharmaceuticals which usually fit the classification of secondary metabolites, strain selection for maximizing yield is important.

The role of secondary metabolites is in most cases not known. It is unlikely that they have no specific function and are simply the result of poor regulation of cellular metabolism. Bu'Lock [1] has suggested that the production of secondary metabolites is a mechanism by which excess intermediates can be metabolized during adverse growth conditions allowing the cell to maintain a functional state. Alternatively, it may be a mechanism whereby excess substrate, e.g. carbohydrate or lipid, may be metabolized. Secondary metabolites with antibiotic activity, though comparatively few in number, may allow the organism to achieve a competitive advantage in nature.

The fungi are sources of secondary metabolites which are invaluable to mankind. Some well-known fungal metabolites include

penicillin, cephalosporin, kojic acid and gibberellic acid. Many other products are known and are compiled in various references e.g. The Pfizer Handbook of Microbial Metabolites by Max W. Miller.

Specific metabolic paths are necessary in achieving the synthesis of these compounds. The biosynthesis of several noted fungal products is outlined in Figure 2 taken from Turner [2].

B. PROBLEM

A mutant strain of the Basidiomycete, Coprinus macrorhizus var. microsporus (Takemaru 5074, ATCC 34960) produces a purple pigment which diffuses through the culture medium. Wild-type strains (Takemaru 5377, 5378) of the same organism do not produce purple pigment under the same conditions. This mutation is quite subtle since there is no nutritional supplement required for the mutant to grow, although pigment production does seem to require that the medium be supplemented with yeast extract [3]. In addition the mutation seems to be recessive since dikaryons (5074 x 5377) and (5074 x 5378) under the same conditions of growth, also do not form the purple pigment.

The purple pigment produced by C. macrorhizus microsporus (5074) may be derived from a primary metabolite produced by both the mutant and wild-type strains and further metabolized by two different pathways or from a secondary metabolite simply metabolized further by some nonspecific enzyme produced only by the mutant. We have noted phenoloxidase activity in the medium of both the wild-type and mutant strains. However in the mutant strain a large increase in phenol-oxidase activity appears concomitantly with purple pigment formation, which usually occurs after eight days growth. The activity in the

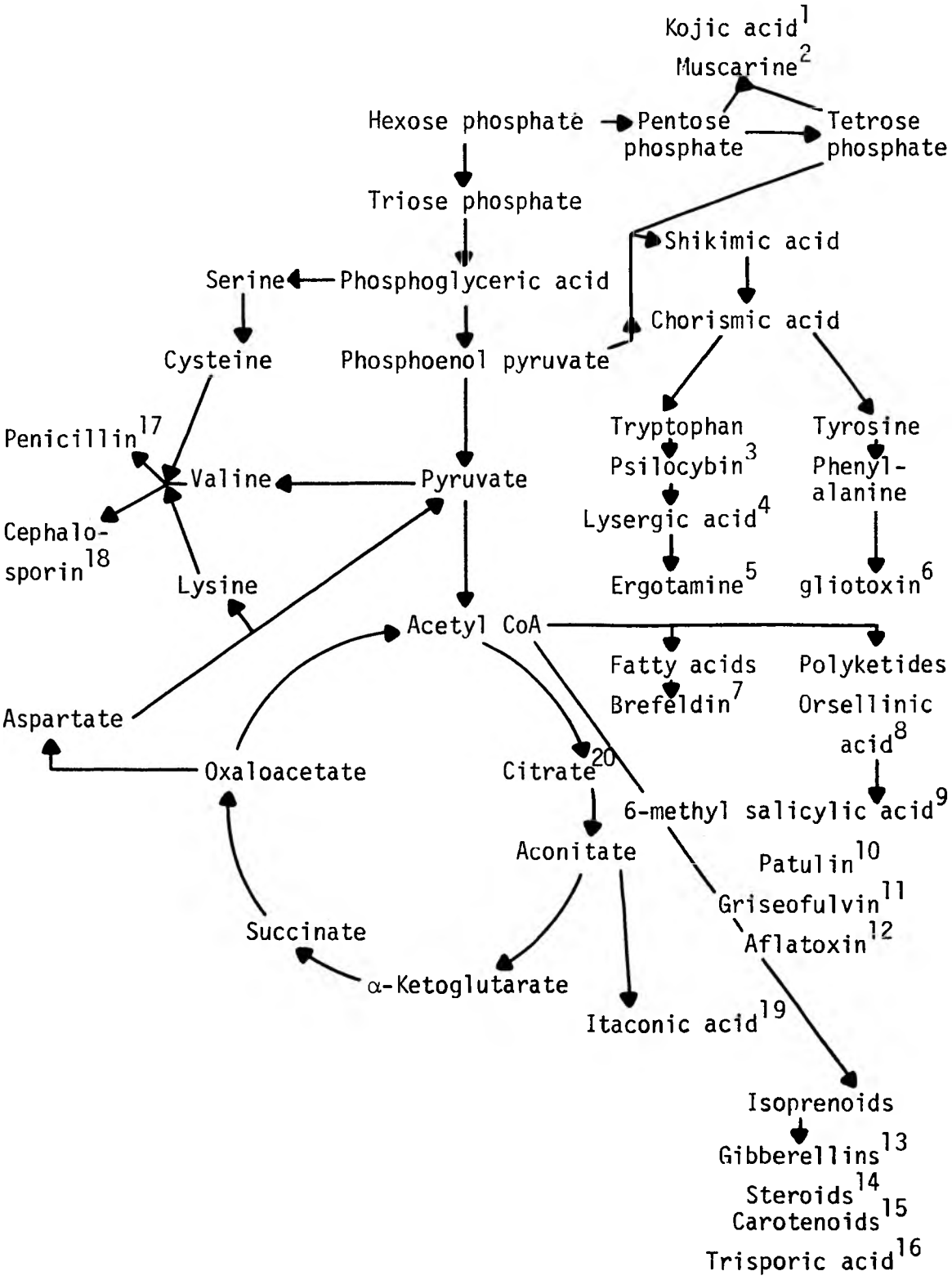


Figure 2. Metabolic Pathways Leading to Secondary Metabolites. (Key, Table I)

TABLE I

KEY TO FIGURE 2

1. *Aspergillus* sp.
2. *Amanita muscaria*
3. *Psilocybe mexicana*
4. *Claviceps* sp.
5. *Claviceps* sp.
6. *Penicillium terlikowski* and other imperfect fungi
7. *Penicillium brefeldianum*, *Nectria radicolata*
8. Widespread
9. Widespread
10. *Penicillium patulum*
11. *Penicillium griseofulvum*
12. *Aspergillus flavus*
13. *Gibberella fujikuroi*
14. Yeast and other fungi
15. Widespread
16. *Blakeslea trispora*
17. *Penicillium chrysogenum*
18. *Cephalosporium acremonium*
19. *Aspergillus niger*
20. *Aspergillus niger*

wild-type remains fairly constant throughout the same growth period. The synthesis of a new protein by the mutant is unlikely. A more probable explanation is that the mutant has lost regulatory control over a constitutive enzyme. Loss of control may occur as the result of any one of the following: 1) a faulty operon controlling the synthesis of enzymes involved in the biosynthesis of a primary metabolite; 2) a defective allosteric protein; 3) the loss of an enzyme in a metabolic pathway that could allow for the accumulation of a metabolite, which is converted to the pigment; 4) drastic alteration of the enzyme-substrate affinity; 5) the loss of a repressor. Studies on these biochemical aspects can only be attempted after a full characterization of the pigment. The goal is two-fold. The characterization of a secondary metabolite as a new compound and elucidating the biochemical difference responsible for its production in the mutant strain.

II. LITERATURE REVIEW

A. FUNGAL QUINONES

A wide variety of quinones are produced by the fungi which range in color from pale yellow to black. Nearly half of the known naturally occurring anthraquinones have been isolated from the fungi and lichens [4]. In Penicillium islandicum, more than twenty anthraquinones have been isolated and characterized [4]. Anthraquinones are distributed fairly widely in molds in contrast to the napthoquinones of which only one representative has been found in the Aspergilli and none in the Penicillia. Fusarium species are frequently brightly colored and at least some of the pigments are napthoquinones. The anthraquinones are quite uncommon in higher fungi [4].

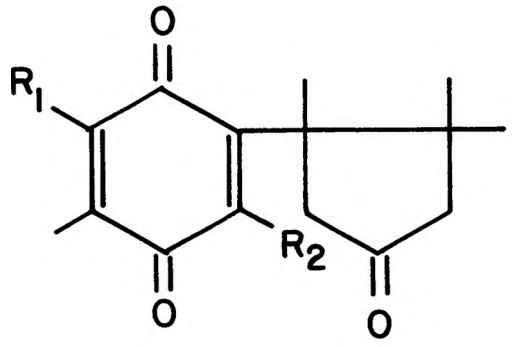
Approximately one-third of the known natural benzoquinones (ca. 90) occur in the fungi and are fairly well distributed among several classes. In the Basidiomycetes, a variety of benzoquinones have been isolated and some of these are tabulated in Table II. As noted, one relatively simple benzoquinone and two related benzoquinones have been isolated from members of the genus Coprinus. Coprinin (2-methyl-5-methoxy benzoquinone) was isolated from the culture medium of Coprinus radians and found to be active as an antibiotic especially against Staphylococcus aureus [5].

More recently, Bollinger [8] isolated and characterized two related benzoquinones which have antibiotic properties from the culture medium of Coprinus lagopus, lagopodin A (IV, Fig. 3) and B (V, Fig. 3). A

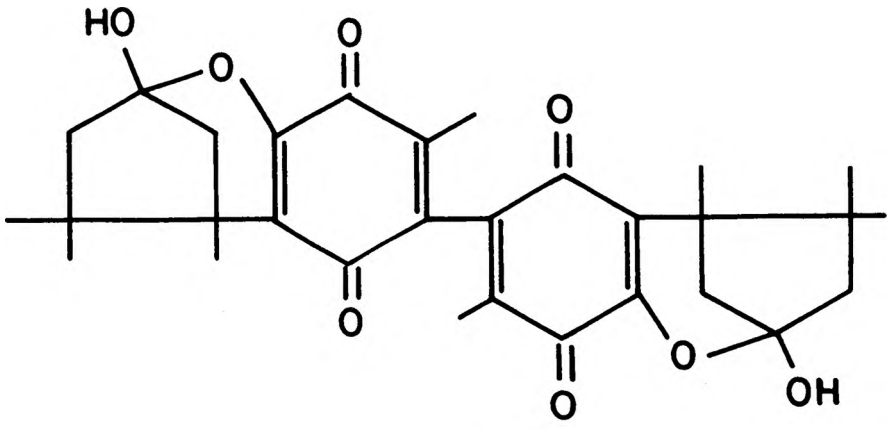
TABLE II

BENZOQUINONES FROM BASIDIOMYCETES

Benzoquinone	Source	Ref. No.
coprinin (2-methyl-5-methoxy benzoquinone)	Coprinus radians	[5]
2,5-dimethoxy benzoquinone	Polyporous tumosus	[6]
3-hydroxy-5-methoxy-2-methyl benzoquinone	Lentinus degener	[7]
lagopodin A and B	Coprinus lagopus	[8]
polyporic acid	Polyporus nidulans	[9]
leucomelone	Polyporus leucomelas	[10]
muscarufin	Amanita muscaria	[11]



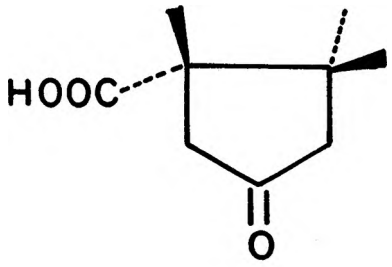
(IV) R_1=R_2=H (V) R_1=H, R_2=OH



(VI)

Figure 3. Structures of Lagopodin A (IV), Lagopodin B (V), and Lagopodin C (VI)

third component, lagopodin C (VI, Fig. 3), was judged to be an artefact of the isolation procedure. The ultraviolet-visible absorption spectrum of lagopodin A is consistent with that of a 2,5- or 2,6-dialkylbenzoquinone. In addition, the infrared spectrum indicated a second carbonyl band at 1745 cm^{-1} which was determined to be the carbonyl absorption of a cyclopentanone ring. On ozonolysis, lagopodin A gave an optically active C_9 acid identified as the enantiomer of the (1R)-acid derived from (+)-camphor (VII, Fig. 4). These results and additional data from nmr spectroscopy and ms suggested the structure (IV) in Figure 3. Lagopodin B is similar to lagopodin A and differs by an additional atom of oxygen, as a hydroxyl group on the benzoquinone nucleus (Fig. 3). The ultraviolet-visible absorption is like that of a 2,5 (or 2,6)-dialkylhydroxybenzoquinone. Oxidation of lagopodin B with alkaline peroxide or ozonolysis gave the same acid (VII, Fig. 4) as that obtained with lagopodin A [8]. The structure (V, Fig. 3) for lagopodin B was supported by these data, however some inconsistencies were observed in the nmr and infrared spectra. In the nmr spectrum, the position of the hydroxyl proton resonance (δ 4.88 ppm) was more characteristic of an aliphatic hydroxyl group than a hydroxyquinone (ca. 7 ppm). Infrared absorption due to the cyclopentanone carbonyl band could only be discerned as a weak shoulder at 1745 cm^{-1} when measured as a 5% solution in carbon tetrachloride. However, on dilution a distinct band appears at 1745 cm^{-1}. These observations lead to the conclusion that lagopodin B has the hemiketal structure



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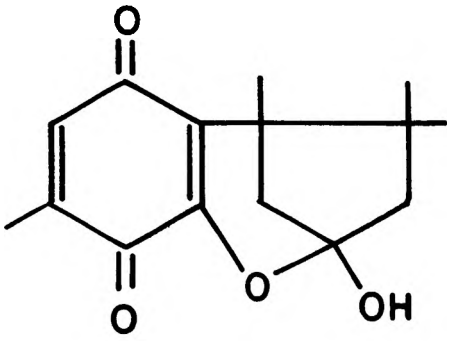

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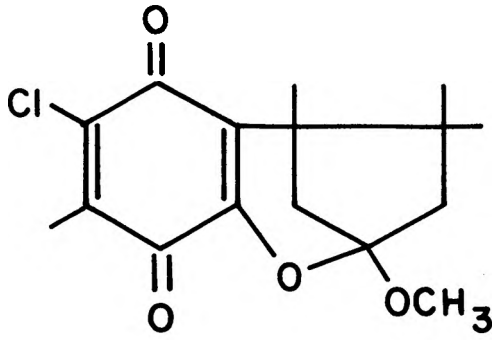
Figure 4. Degradation Product of Lagopodins (VII) and the Structure of Lagopodin B as a Hemiketal (VIII)



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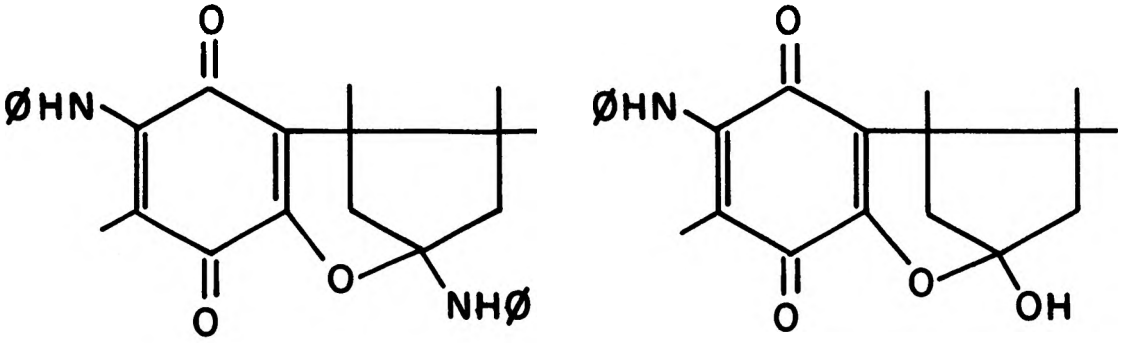


(X)

Figure 5. Derivatives of Lagopodin B

(VIII, Fig. 4) that is in equilibrium with the open form in solution. An unusual methyl ketal derivative was formed on reaction with ferric chloride and methanol. The structure (IX, Fig. 5) was assigned based on nmr data. Also, lagopodin B reacted with aniline to give two products, a normal anilino derivative (XI, Fig. 5) and a dianilino compound (X, Fig. 5). These structures also support the conclusion that lagopodin B exists as a hemiketal structure (VIII, Fig. 4).

When lagopodin B is treated with aqueous sodium hydroxide under mild conditions, it is partly converted to the dimer lagopodin C. It can be degraded to the acid (VII, Fig. 4) and forms a bis-methyl ketal on treatment with ferric chloride in methanol. The molecular weight and the presence of an M+4 peak in the mass spectrum suggests that lagopodin C is a biquinone and the dimer is symmetrical as indicated by the nmr spectrum.

A majority of the fungal benzoquinones appear to be formed by the acetate-malonate pathway [4]. In some cases, the benzoquinone nucleus is derived from shikimic acid through the phenolic intermediate. Some organisms may utilize both the shikimate and the acetate-malonate routes for the biosynthesis of quinones. The polyprenyl side chains of ubiquinones are derived from mevalonate as expected. However, labelling studies show that acetate can only be incorporated into the polyprenol side chain and not into the benzoquinone nucleus. Highly specific incorporation into the benzoquinone ring was found with labelled p-hydroxybenzoic acid [12] and it has been shown that this metabolite arises from the shikimic acid pathway [13]. Helicobasidin

is the only terpenoid quinone whose origin from mevalonate has been established [14]. It is a sesquiterpenoid quinone similar in structure to hydroxylagopodin B and differs only by the substitution of an oxygen atom on the cyclopentane-like substituent at position 2 of the benzoquinone ring. It is expected that these quinones are formed by the same biosynthetic pathways.

B. PHENOLOXIDASES (TYROSINASES, LACCASES)

Tyrosinase (o-diphenol: O_2 oxidoreductase, EC 1.10.3.1) has been shown to be present in a wide variety of organisms. It has been isolated and purified from the common mushroom, Agaricus bisporus [15,16], the Ascomycete, Neurospora crassa [17], Aspergillus nidulans [18], Pseudomonas melanogenum [19], and hamster melanoma [20]. Tyrosinases are unique in that they are bifunctional, possessing two different types of catalytic activity. They are capable of oxidizing catechol or catechol-like compounds to give o-quinones and ortho-hydroxylation of phenols to give catechols. These types of activity have also been referred to as the catecholase and cresolase activities respectively, since the substrates must commonly employed were p-cresol and catechol. The conversion of tyrosine to melanin is in part catalyzed by tyrosinase. The reactions catalyzed are:

(1) phenol hydroxylase function

$$\text{tyrosine} \xrightarrow{\frac{1}{2}\, O_2} \text{dopa}$$

(2) polyphenol oxidase function

$$2\ \text{dopa} \xrightarrow{O_2} 2\ \text{dopaquinone} + 2\ H_2O$$

Several spontaneous reactions follow which ultimately result in the formation of melanin.

(3) dopaquinone ———> leukodopachrome

(4) leukodopachrome + dopaquinone ——> dopachrome + dopa

(5) dopachrome ———> melanin (several steps)

Laccase (p-diphenol: oxygen oxidoreductase, EC 1.10.3.2) is known to occur in several species of Basidiomycetes and Ascomycetes, both as an intracellular and extracellular enzyme [21]. Laccases are also capable of catalyzing the conversion of dopa to dopaquinone and ultimately dopachrome. They are not capable of catalyzing hydroxylation of phenol or phenol-like substrates. Phenoloxidase activity, therefore, may represent tyrosinase, laccase or perhaps some other enzyme activity capable of oxidizing dopa to dopaquinone. In many cases, the phenoloxidase activity has not been studied as to its hydroxylation capability especially since the widely used spectrophotometric assay for phenoloxidase activity is based on the increase in absorbance at 475 nm due to the formation of the red-colored product, dopachrome.

Phenoloxidases have been implicated in various aspects of development in fungi. The development of protoperithecia in Neurospora crassa was correlated with high tyrosinase activity [22]. Horowitz, et al. [23] found that N. crassa mutants unable to form protoperithecia or exhibit tyrosinase activity could be induced to

form active tyrosinase with no effect on female sterility and concluded that the characteristics were related through a common biochemical defect. In Podospora anserina, Esser [24] suggested that increased laccase activity and perithecial development are probably related.

Fruit-body formation and phenoloxidase activity have been closely correlated in the basidiomycete, Schizophyllum commune [25]. This conclusion was supported by three lines of evidence: (1) Inhibitors of phenoloxidase activity influenced both quantitative and qualitative aspects of fruit-body formation, (2) phenoloxidase activity was clearly demonstrated in fertile mycelia and shown to be absent in sterile mycelia, (3) only the cell-free extracts of fertile mycelia were capable of oxidizing dopa indicating phenoloxidase activity. Extracts of fruiting bodies were also found to contain phenoloxidase activity. In a later study, Leonard and Phillips [26] established that phenoloxidase activity increases until the formation of mature fruiting-bodies and subsequently decreases. Schizophyllum commune requires light for the complete fruiting response and in dark-grown colonies, morphogenesis proceeds until the formation of compact masses of hyphae. To this developmental stage, phenoloxidase activity increased similarly. Phenoloxidase inhibitors also drastically reduced the formation of the initial hyphal aggregates suggesting phenoloxidase activity is a consequence of or is concomitant with the early morphogenetic response. The relationship of phenoloxidase activity to development in the common mushroom Agaricus bisporus has also been studied [27] but these studies are hampered since fruiting on

synthetic medium has not been accomplished with this organism. The presence and absence of laccase and tyrosinase activities at different stages of the life cycle of A. bisporus was established [27]. Laccase activity characterizes the vegetative stage and tyrosinase activity develops at the time of initiation and development of fruit bodies. The life-cycle of all Coprinus species differs from that of other Basidiomycetes, for sporulation is accompanied by autolysis of the basidiocarp to form a black amorphous mass. The black pigmentation is due to melanin-containing spores and this may be related perhaps to an increased activity of phenoloxidase during fruit-body formation and the subsequent autolysis. Various aspects of development have been studied [28-35] in C. macrorhizus microsporus, however the relationship of phenoloxidase activity to development in this organism and other Coprinus species remains obscure.

Uno and Ishikawa [31] isolated fruiting body-inducing substances (FIS) which were effective in inducing fruiting bodies in monokaryotic mycelia of C. macrorhizus microsporus. These substances were identified as adenosine-3'-monophosphate, adenosine 3',5'-cyclic monophosphate (cyclic AMP) and a protein which is bound with the cyclic AMP. A subsequent study [32] reported on the detection of adenyl cyclase and phosphodiesterase activities in mycelia of strains of C. macrorhizus microsporus which form fruiting bodies and these activities were not found in those which do not form fruiting bodies. These enzymes may play an important role in regulating the concentration of cyclic AMP in vivo. Cyclic AMP has been found to induce the synthesis of tyrosinase in Neurospora crassa [36]. Tyrosinase

activity, as stated earlier, increases in sexually differentiating cultures of N. crassa. Although the relationship between cyclic AMP, tyrosinase, and development has not been studied in any one organism, it is logical that these aspects may be intimately related.

III. EXPERIMENTAL

A. ISOLATION AND CHARACTERIZATION OF HYDROXYLAGOPODIN B AND LAGOPODIN B

The following describes cultural conditions and experimental methods used in obtaining hydroxylagopodin B and lagopodin B.

1. Organisms. Coprinus macrorhizus microsporus (5074, 5377, 5378) were provided by Dr. Philip G. Miles, Dept. of Biology, State University of New York, Buffalo, NY who originally obtained the culture through the courtesy of Dr. Tsuneo Takemaru, Dept. of Biology, Okayama University, Okayama, Japan.

2. Media and Cultural Conditions. C. macrorhizus microsporus was cultured on complete media [37] supplemented with yeast extract. The medium contained: 20.0 g glucose, 2.0 g peptone, 0.5 g yeast extract, 0.46 g KH_2PO_4, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1000 ml glass-distilled H_2O. Media (4 liters) was inoculated with a suspension of mycelial fragments obtained by homogenization of mycelial growth from a 10-day-old plate with 10-20 mls sterile H_2O. For the isolation of hydroxylagopodin B and lagopodin B the cultures were incubated with shaking at 22°C for 12 days. Purple pigment formation in strain 5074 usually began after 8 days incubation with shaking. The fruiting medium was prepared as follows: 100 g of fresh potatoes and 10 g sucrose were homogenized in a Waring blender at low speed. Agar (10 g) was added to the homogenate and taken up to 500 mls with glass-distilled H_2O, autoclaved, and poured into large petri dishes.

3. Isolation of Hydroxylagopodin B from Mutant Strain 5074. The mycelia were removed by vacuum filtration on two layers cheese-cloth. Cell-free medium (4 liters) was acidified with 2N HCl to a

pH ~ 3 and extracted with ethyl acetate (500 mls) using a liquid-liquid extraction apparatus. The ethyl acetate extract was reduced to 100 mls by vacuum evaporation at 40°C. The pigment was extracted with 0.1M $NaHCO_3$ (2 x 100 mls), acidified with 2N HCl (pH ~ 3), and re-extracted into benzene. Following evaporation of this extract to near dryness, the residue was taken up in 2% MeOH-$CHCl_3$ (v./v.) and separated on a silica-gel G column (3 x 40 cm) eluted with 2% MeOH-$CHCl_3$ (v./v.). Each fraction was dried over Na_2SO_4. Three fractions were collected listed in order of elution: 1) a yellow band 2) a purple band and 3) a purple band larger than the two previous bands. A large amount of brown material remained adsorbed near the top of the column. Fraction 2 was evaporated in vacuo at 40°C leaving a yellow-brown syrupy residue. Bright orange crystals formed upon crystallization and recrystallization from a benzene-heptane mixture. The crystalline compound was dried 2 hrs in vacuo at 80°C using an Abderhalden apparatus (yield 14 mg., mp 184-186°C).

4. Isolation of Lagopodin B from Wild-type Strain 5377.

Lagopodin B was isolated according to the method of Bollinger [8] modified in part. Three liters of filtered spent medium were extracted using a separatory funnel with 1 liter. diethyl ether (2x) made peroxide-free by washing with ferrous sulfate solution. The lemon-yellow ether extract was evaporated to dryness under vacuum at 40°C. The residue (400 mg) was taken up in 100 mls of diethyl ether and extracted 2x with 100 mls 0.5M Na_2CO_3 solution. The carbonate extracts,which were intensely violet in color, were combined and immediately neutralized with 5M H_2SO_4, taking on a yellow color. The

yellow pigment was extracted into benzene (150 mls, 2x); the benzene layer was washed with two 75 mls portions of distilled H_2O, dried over Na_2SO_4 and concentrated to dryness under vacuum at 40°C. The partially purified product (164 mg) was taken up in $CHCl_3$, applied to a silica gel 60 column (13 x 2.5 cm) and eluted with 2% MeOH-$CHCl_3$ (v./v.). Two major components were separated appearing as brown bands on the column. Lagopodin B was eluted as fraction I (first brown band). Fraction I was evaporated in vacuo at 40°C. Crystalline material was recovered on evaporation. The product was recrystallized from a diethyl ether-heptane mixture and dired on a porous plate (yield 107 mg, mp 109-111°C, lit. mp 110-112°C).

5. Physical Methods. Melting points were taken on a Kofler hot stage. Ultraviolet-visible spectra were measured in 95% ethanol using a Beckman spectrophotometer. Infrared spectra were determined in a KBr matrix with a Perkin-Elmer model 337 spectrophotometer. Mass spectra were determined on a CEC-21-110 or a JEOL DMS-100 by direct sample introduction. Analytical tlc was carried out on silica gel G or 60 (E. Merck) layers using 2% MeOH-$CHCl_3$ as the solvent system.

B. ISOLATION AND CHARACTERIZATION OF PHENOLOXIDASE

The following describes the isolation procedure and the characterization of the phenoloxidase from strain 5074.

1. Culture Conditions. Coprinus macrorhizus microsporus (5074) was cultured on complete medium [37] supplemented with yeast extract. The medium was inoculated with a suspension of macerated mycelia and incubated on a shaker at room temperature (24°C).

2. Enzyme Isolation and Purification. Two Fernbach culture flasks each containing 1 liter modified medium were inoculated with

mutant strain C. macrorhizus microsporus (5074, ATCC 34960) and incubated for 23 days. The cells were harvested by centrifugation at 5,000 g. and discarded. Phenoloxidase which occurs in the culture medium was precipitated in the cold with ammonium sulfate to 50% saturation. After 30 min., the red-brown precipitate was collected by centrifugation at 10000 g. and dissolved in a suitable amount of 0.05M phosphate buffer, pH 7.2. Dialysis against 0.01M phosphate buffer, pH 7.2, was carried out overnight with one change of buffer after six hours. An ECTEOLA (epichlorohydrin triethanolamine) cellulose column was prepared by adjusting the pH of a water suspension to 7.2 and equilibrating overnight with 0.05M phosphate buffer, pH 7.2. The dialyzed enzyme solution was applied to the column and fractions (10 ml) collected which were assayed for protein content at 280nm and enzyme activity. Active fractions with 0.1 absorbance or greater were pooled and the enzyme precipitated again with ammonium sulfate to 60% saturation. A lemon-yellow precipitate was collected by centrifugation at 17000 g. and dissolved in a minimum amount of 0.05 M phosphate buffer, pH 7.2.

3. Assay and Definition of the Enzyme Unit. The phenoloxidase was assayed by following the appearance of dopachrome spectrophotometrically at 475 nm [20]. An aliquot of enzyme solution was diluted to 4 ml. with 0.05 M phosphate buffer, pH 7.2, and equilibrated 5 min. at 30^{o}C. At time zero, 1 ml. of dopa solution (4 mg/ml) was added and the absorbancy measured. After 5 min. of incubation at 30^{o}C, a second reading was taken. The enzyme concentration is proportional

to the change in absorbancy. An enzyme unit is defined as an absorbance change of 0.001 min^{-1} under the above conditions [25]. Protein concentrations were measured by the method of Lowry [38] with crystalline bovine serum albumin as standard. Specific activity is expressed as enzyme units per mg protein.

4. Kinetic and Inhibition Studies. Average velocities were measured using the normal assay procedure and varying the concentration of dopa. The same enzyme dilution and substrate dilutions were used for the inhibition studies. Cysteine, which is known to inhibit phenoloxidases, was used for the inhibition studies. Inhibitor solutions and appropriate dilutions were made using phosphate buffer.

5. Disc Gel Electrophoresis. Disc gel electrophoresis was carried out according to the procedure of Davis [39]. Coumassie Blue rather than Amido Black was used for the detection of protein bands. Active bands were detected by immersing the gel in dopa solution of normal assay concentration.

IV. RESULTS AND DISCUSSION

A mutant strain of Coprinus macrorhizus microsporus (5074, ATCC 34960) excretes into the medium a purple pigment which appears regularly after 8 days incubation with shaking at 22°C. A wild-type strain of C. macrorhizus microsporus (5377) incubated under the same conditions does not produce the purple pigment, but rather a yellow pigment. These observations also occur when the strains are cultured on solid agar medium at room temperature. Mycelial growth for both strains is more rapid at 37°C. However, in the mutant strain, purple pigment does not form at elevated temperatures, suggesting perhaps that the enzyme(s) responsible for the production of purple pigment may be thermally inactivated. The two strains are compatible and undergo dikaryotization followed by fruit-body formation within 10 days when cultured on potato-sucrose medium [28]. The fruiting-body undergoes autolysis within six hours forming a black morphous mass characteristic of the genus Coprinus; the exudate contains black spores. In Coprinus cinereus (=lagopus), the optimum temperature for mycelial growth is about 37°C, but fruiting bodies are not formed above about 30°C [40]. The dikaryon 5074 x 5377 does not form pigment on solid medium.

The culture medium of both strains was assayed for phenoloxidase (PO) activity at various times of growth (Fig. 6). A large increase in phenoloxidase activity is noted at 8-days growth in the mutant strain (5074), concomitant with purple pigment formation, whereas in the wild-type strain (5377), phenoloxidase activity remains constant

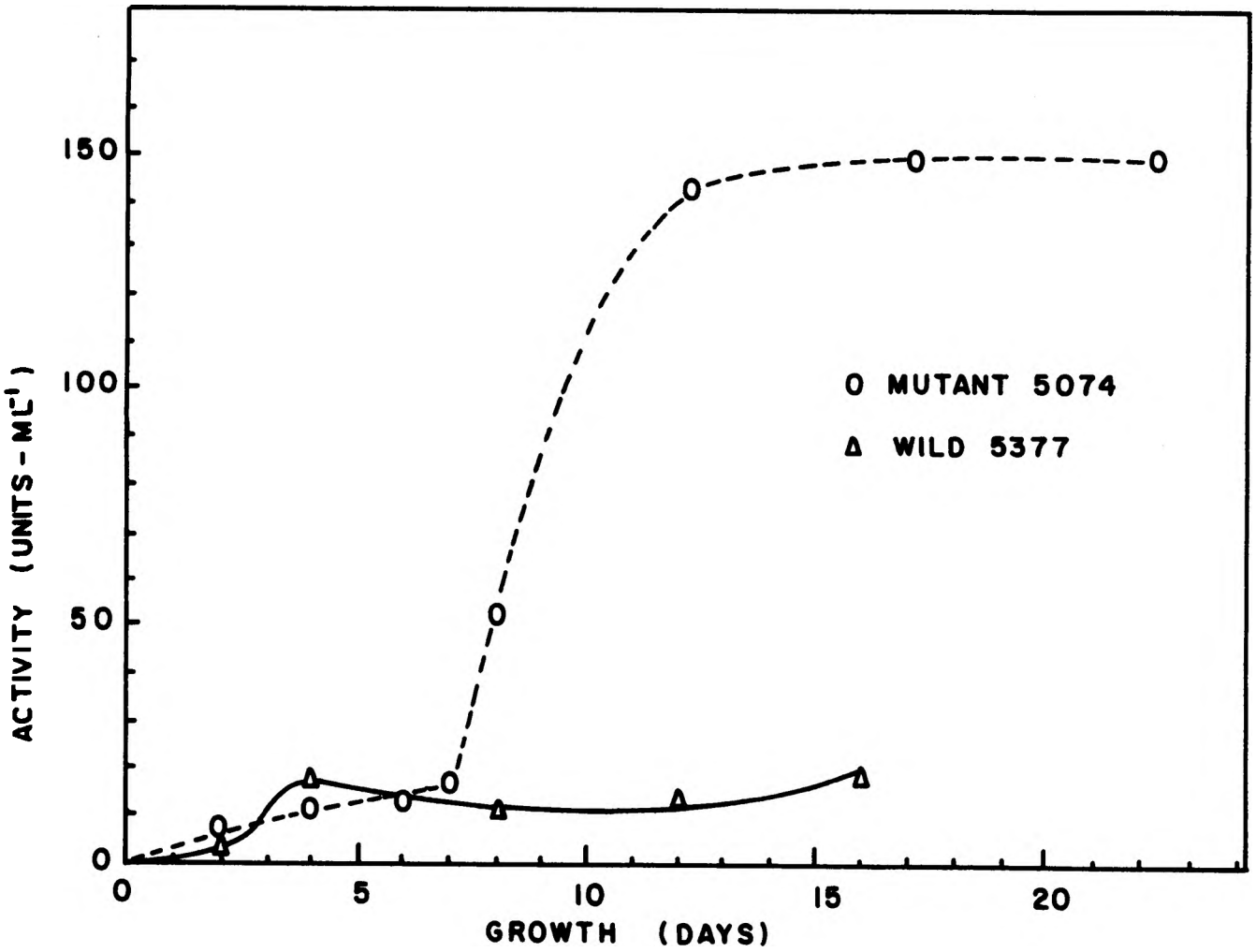


Figure 6. Phenoloxidase Activity of the Culture Medium as a Function of Age

throughout the same period. The increase in phenoloxidase activity seems to parallel pigment formation; the pigment may be the direct result of non-specific activity of the phenoloxidase.

The approach followed in elucidating the nature of the mutation in strain 5074 was to isolate and characterize (A) the purple pigment produced by the mutant, (B) the "normal" pigment from the wild-type strain and (C) the phenoloxidase expressed by the mutant. Since phenoloxidases have been implicated in the development processes of Basidiomycetes [25-27,41], it is possible that the phenoloxidase activity expressed in the mutant would not normally manifest itself at this stage of development. In Coprinus, the rather sudden autolysis of the fruiting body accompanied by the formation of melanin and melanin-containing spores most likely involves the enzyme phenoloxidase, probably a tyrosinase.

A. HYDROXYLAGOPODIN B FROM C.MACRORHIZUS MICROSPORUS (5074)

The organism was grown in stationary culture at 21°C for one month. When the ethyl acetate extract of the filtered-spent medium was chromatographed on silica-gel thin layer plates developed with 2% MeOH-$CHCl_3$, a series of seven individual pigments can be resolved. The crude ethyl acetate extract was chromatographed on a silica gel column and eluted with 2% MeOH-$CHCl_3$, yielding seven pigmented fractions. Ultraviolet spectra of the isolated components indicated a structural similarity by the fact that each exhibited a single absorption maximum in the range 260-320 nm. The pigments may differ only by the nature and number of substituents on a common structure. Table III

gives R_f and UV spectral data for each component. In shake cultures, C. macrorhizus microsporus produces purple pigmentation within 8 days and becomes very intense at 10 days. Thin-layer chromatography of the crude ethyl acetate extract on silica gel indicated the presence of four components: 1) yellow, 2) blue-violet, 3) violet, 4) maroon. A considerable amount of brown residue remained at the origin. A much larger concentration of violet pigments was present in shake cultures as indicated by the intensity in pigmentation of the medium and violet spots on thin-layer chromatograms. Our interest was directed toward the structural characterization of the purple pigments and it was evident that shake cultures afforded a higher concentration of these compounds.

The isolation and characterization of hydroxylagopodin B has been described previously [42]. The purified crystalline hydroxylagopodin B gives a positive $FeCl_3$ reaction characteristic of phenolic compounds. Lagopodin B and C also give positive $FeCl_3$ tests [8]. The ultraviolet and infrared spectra of hydroxylagopodin (Figs. 7 and 8) are consistent with a 2,5-dihydroxy-3,6-dialkyl benzoquinone structure. Table IV lists UV λ_{max} and IR carbonyl absorption for various 2,5-dihydroxybenzoquinones. The structure of helicobasidin (Fig. 9, XII) differs only slightly from hydroxylagopodin B, specifically by the substitution of an oxygen atom on the cyclopentane-like nucleus at the 6 position of the parent benzoquinone ring. It gives a violet solution in aqueous sodium carbonate [4]. Hydroxyperezone (Fig. 9, XIII) has a similar structure and the spectral data are also

TABLE III

ULTRAVIOLET SPECTRAL DATA FOR PIGMENTS ISOLATED FROM STATIONARY CULTURES

Pigment	R_f[a]	UV λ_{max} (nm)	Comments
1 (yellow)	0.81	264	
2 (pink)	0.79	301	
3 (pink)	0.74	317	
4 (yellow)	0.70	263	Lagopodin B
5 (pink)	0.65	298	
6 (purple)	0.56	306	Hydroxylagopodin B
7 (yellow)	0.48	293	

[a]On Silica gel G (E. Merck) thin-layers with 2% MeOH/$CHCl_3$ as solvent system.

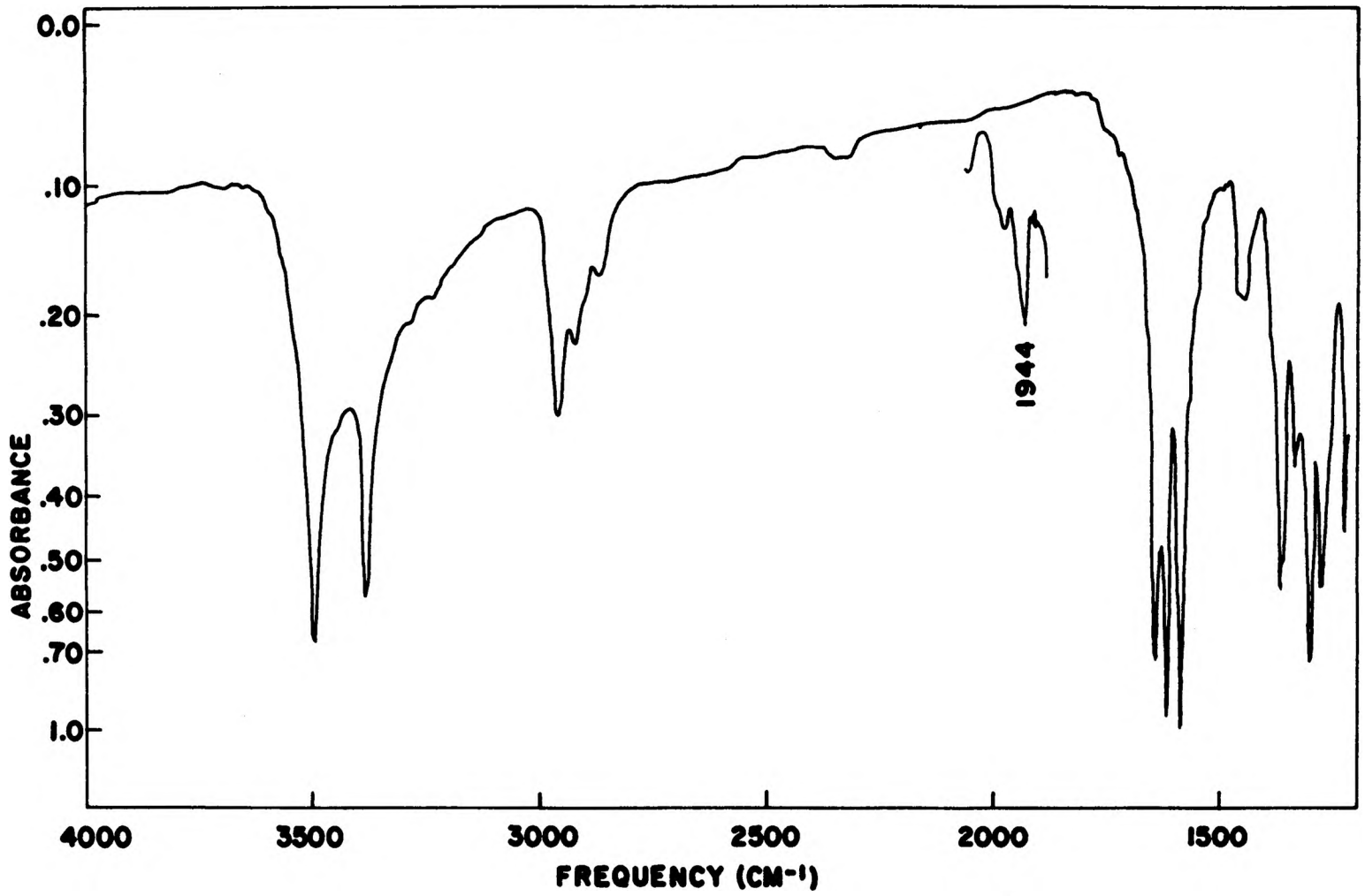


Figure 7. Infrared Spectrum of Hydroxylagopodin B, 4000-1200 cm^{-1} (KBr matrix)

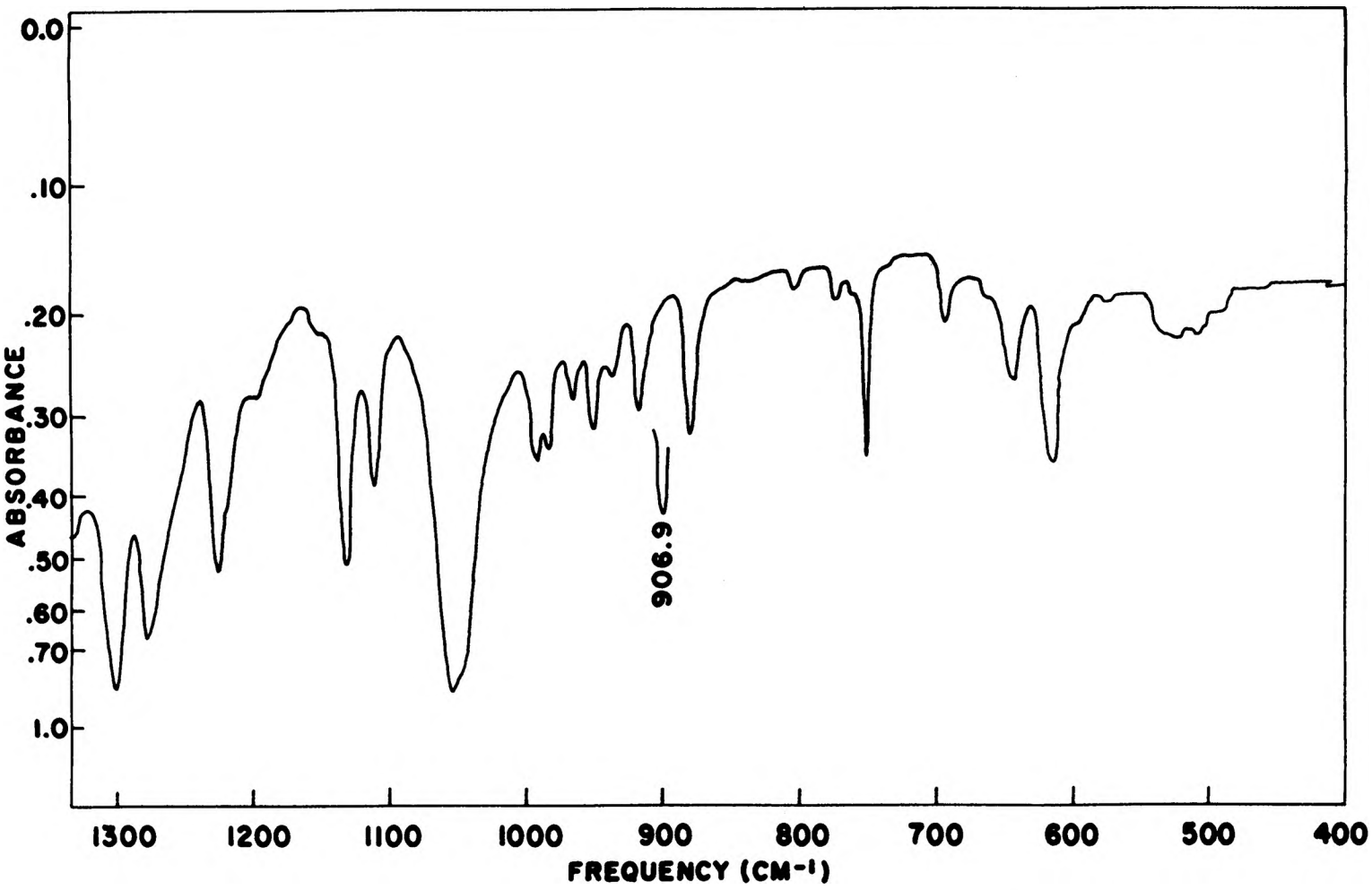
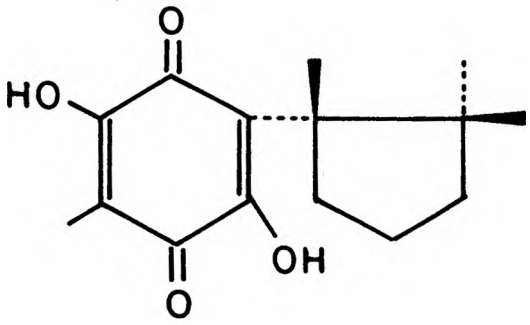


Figure 8. Infrared Spectrum of Hydroxylagopodin B, 1350-400 cm^{-1} (KBr matrix)

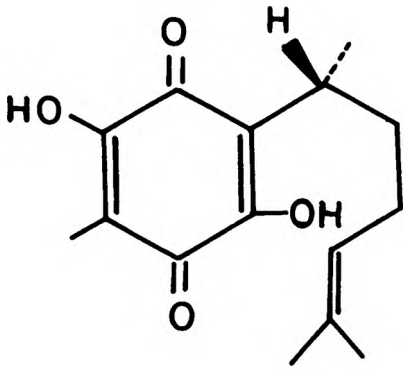
TABLE IV

ULTRAVIOLET MAXIMA AND CARBONYL INFRARED ABSORPTION FOR VARIOUS 2,5-DIHYDROXYBENZOQUINONES

Quinone	UV λ_{max} (nm)	ν_{CO} (cm^{-1})
2,5-dihydroxy benzoquinone	279, 286	1646, 1620
3,6-dihydroxy thymoquinone	293	1616
spinulosin (2,5 dihydroxy-3-methoxy-6-methyl benzoquinone)	297	1655, 1637
hydroxyperezone	295	1640, 1615
helicobasidin	297	1609
bhogatin	295	1635
hydroxylagopodin B	307	1650, 1627



(XII)



(XIII)

Figure 9. Structures of Helicobasidin (XII) and Hydroxyperezone (XIII)

consistent with a 2,5-dihydroxy-3,6-dialkyl benzoquinone structure. Helicobasidin is a cyclic isomer of hydroxyperezone (Fig. 9).

Hydroxylagopodin B has a molecular formula, $C_{15}H_{18}O_5$ (M^+ 278), as determined by high resolution mass spectroscopy. Hydroxylagopodin B was clearly related to lagopodin B as evidenced by the fragmentation pattern (Figs. 10 and 11). The most abundant ion and molecular ion (M^+) differed by 16 mass units, suggesting oxygen atom substitution on the benzoquinoid nucleus.

With these data, pigments 4 and 6 (Table III), isolated from stationary culture can be identified as lagopodin B and hydroxy-lagopodin B respectively. From the UV data, pigments 2, 3, 5 and 7 are probably 2,5-dihydroxy-3,6-dialkyl benzoquinones like hydroxy-lagopodin B. The pigments probably differ by the nature of the alkyl substituents on the benzoquinone nucleus. Pigment 1 is probably like lagopodin B i.e. a dialkylhydroxybenzoquinone structure. Thus, the purple pigmentation of mutant strain (5074) medium represents a series of co-metabolites, which are probably 2,5-dihydroxy-3,6-dialkylbenzoquinones. One of these has been identified as hydroxy-lagopodin B.

B. LAGOPODIN B FROM C. MACRORHIZUS MICROSPORUS (5377)

Crystalline product obtained from the filtered-spent medium of C. macrorhizus microsporus (5377) was identified as lagopodin B on the basis of melting point, co-chromatography with standard lagopodin B obtained from P. Bollinger, the violet color reaction on treatment with base and the mass spectrum (Fig. 11).

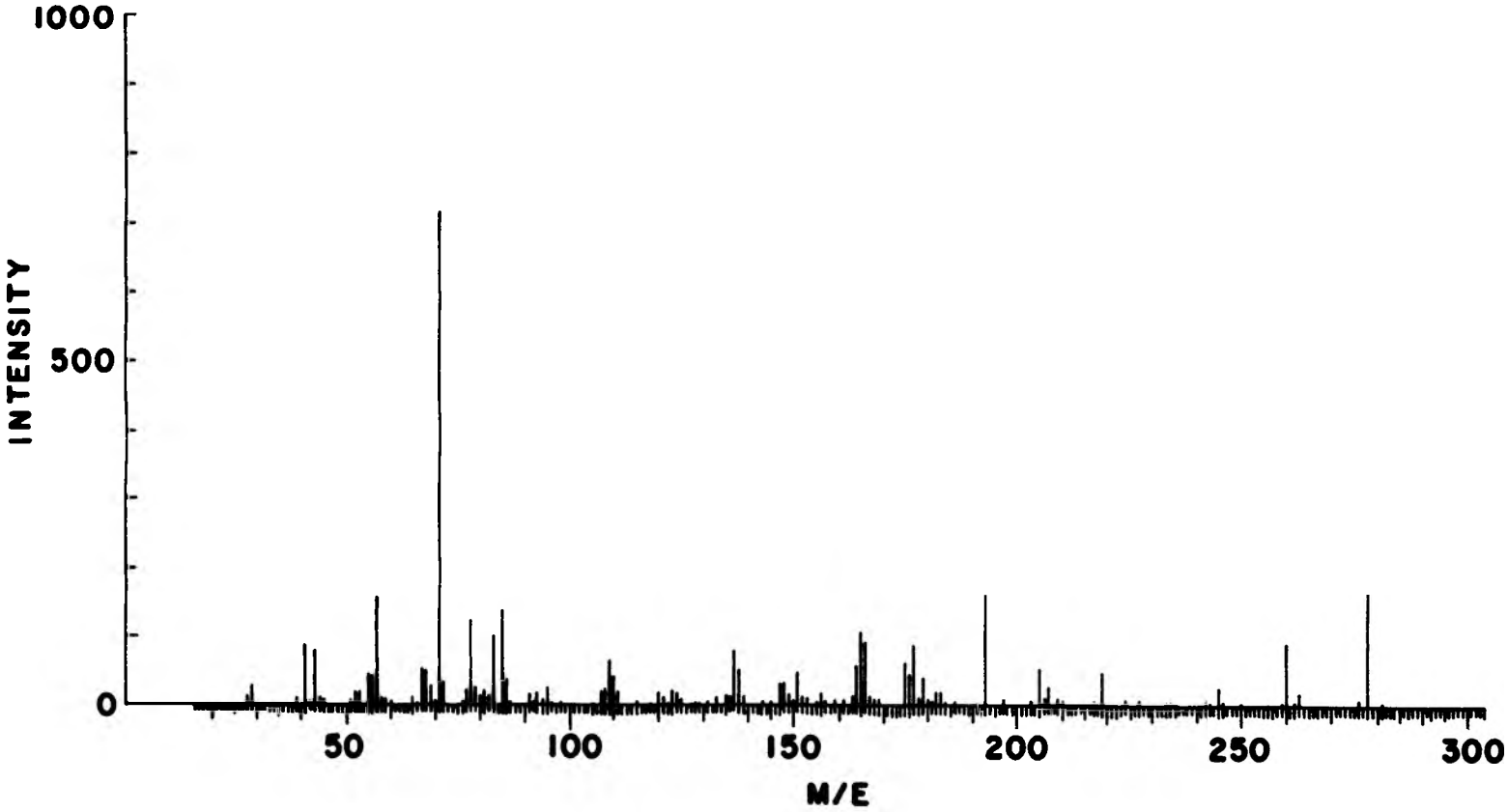


Figure 10. Mass Spectrum of Hydroxylagopodin B

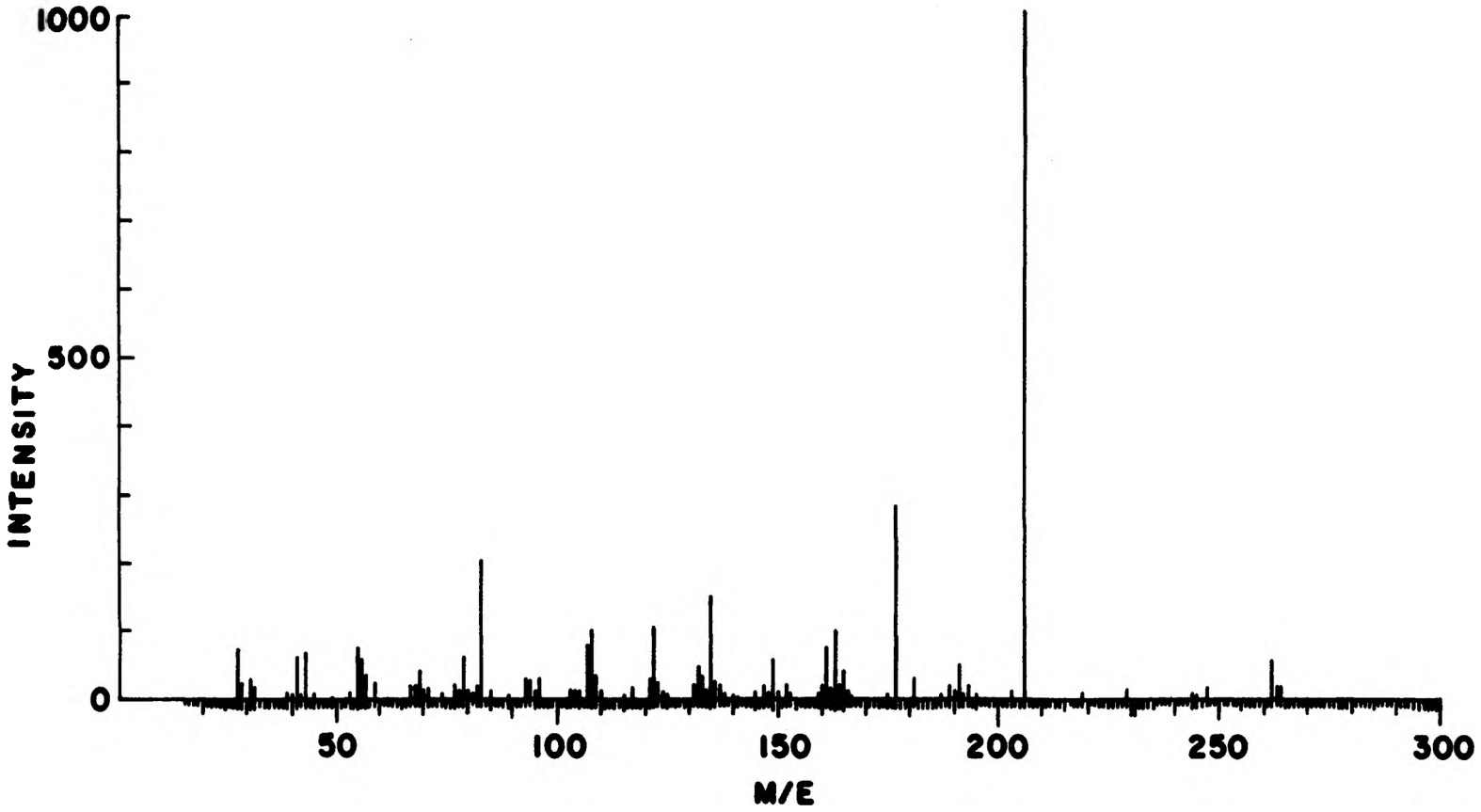


Figure 11. Mass Spectrum of Lagopodin B

The mass spectrum gives a molecular ion of m/e 262 as expected, however, the most abundant ion occurs at m/e 206. Bollinger [8] reports the most abundant at m/e 178 and the difference may be explained by a different ionization potential used in obtaining the mass spectrum. A different fragmentation pattern would be expected if a significantly different ionization potential is used; the molecular ion should be the same.

The yield of lagopodin B was 33 mg/l culture medium and compares well to the yield from C. lagopus, 26 mg/l [8]. Lagopodin B may be a characteristic secondary metabolite of Coprinus useful in the classification of members of this genus.

C. PHENOLOXIDASE FROM C. MACRORHIZUS MICROSPORUS (5074)

The isolation and purification of the extracellular phenoloxidase from mutant Coprinus macrorhizus microsporus (5074) was carried out two times. Enzyme purification for each isolation are summarized in Tables V and VI. Similar results were obtained in each case, however in isolation 2 (Table VI), the final purification step included the removal of non-active protein by a 0-40% $(NH_4)_2SO_4$ fractionation. The enzyme was precipitated as the 40-60% $(NH_4)_2SO_4$ fraction and a higher specific activity was obtained in the final preparation. In isolation 2 a much higher overall yield was obtained since a large loss in activity was experienced in the prolonged dialysis step of isolation 1. It is apparent that in both cases very little purification was achieved by weak anion exchange (ECTEOLA) chromatography.

TABLE V

SUMMARY OF PHENOLOXIDASE PURIFICATION-ISOLATION NO. 1

	Step	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification
1.	Crude medium	1,780	123,000	6,330	19.4	100	--
2.	50% $(NH_4)_2SO_4$ precipitate	333	57,800	200	289	47	15
3.	Dialysis[1]	215	6,810	184	37	5.5	1.9
4.	ECTEOLA-column eluate	306	7,660	23.0	333	6.2	17
5.	60% $(NH_4)_2SO_4$ precipitate	30	2,300	3.1	742	1.9	38

[1]Storage for 5 days at 4°C resulted in considerable loss of activity.

TABLE VI

SUMMARY OF PHENOLOXIDASE PURIFICATION-ISOLATION NO. 2

	Step	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification
1.	Crude medium	3500	381,000	18,000	50	100	--
2.	50% $(NH_4)_2SO_4$ precipitate	170	142,000	301	472	37	9.4
3.	Dialysis	230	148,000	398	372	39	7.4
4.	ECTEOLA-column	495	138,000	248	556	36	11.1
5.	40-60% $(NH_4)_2SO_4$ precipitate	13	96,000	87	1100	25	22.0

The kinetics of the Coprinus phenoloxidase were studied by graphical analysis. A double reciprocal plot of the kinetic data gave a straight line (Fig. 12) indicative of normal Michaelis-Menten kinetics. These data were fit to the best straight line by the least squares method. This analysis gave a K_m of 2.33 mM using D,L-dopa as the substrate and a V_{max} of 52.2 units/min. The K_m is a characteristic parameter for a given enzyme and is useful for making comparisons between enzymes from different sources. Michaelis-Mentenconstants (K_m) for phenoloxidases from other organisms are tabulated in Table VII and it is apparent that the value obtained for C. macrorhizus microsporus phenoloxidase compares well to other phenoloxidases especially laccase from S. commune. Tyrosinases seem to exhibit a higher affinity for dopa as their Km's are somewhat lower. The Coprinus phenoloxidase may be a laccase since tyrosinases of fungi are typically endoenzymes and are not excreted into the culture medium [43].

Fling et al. [17] found that phenoloxidase (a tyrosinase) from N. crassa was strongly inhibited by cysteine. Cysteine inhibited phenoloxidase from C. macrorhizus microsporus. The mechanism of inhibition is believed to be complexation with the cuprous ion present in the enzyme. Inhibition of this type is expected to be non-competitive in nature and this was found to be the case (Fig. 13) with the Coprinus phenoloxidase. Tyrosinases and laccases are copper-containing enzymes and hence inhibition by cysteine is expected in both cases. The K_i for cysteine is 25 μM.

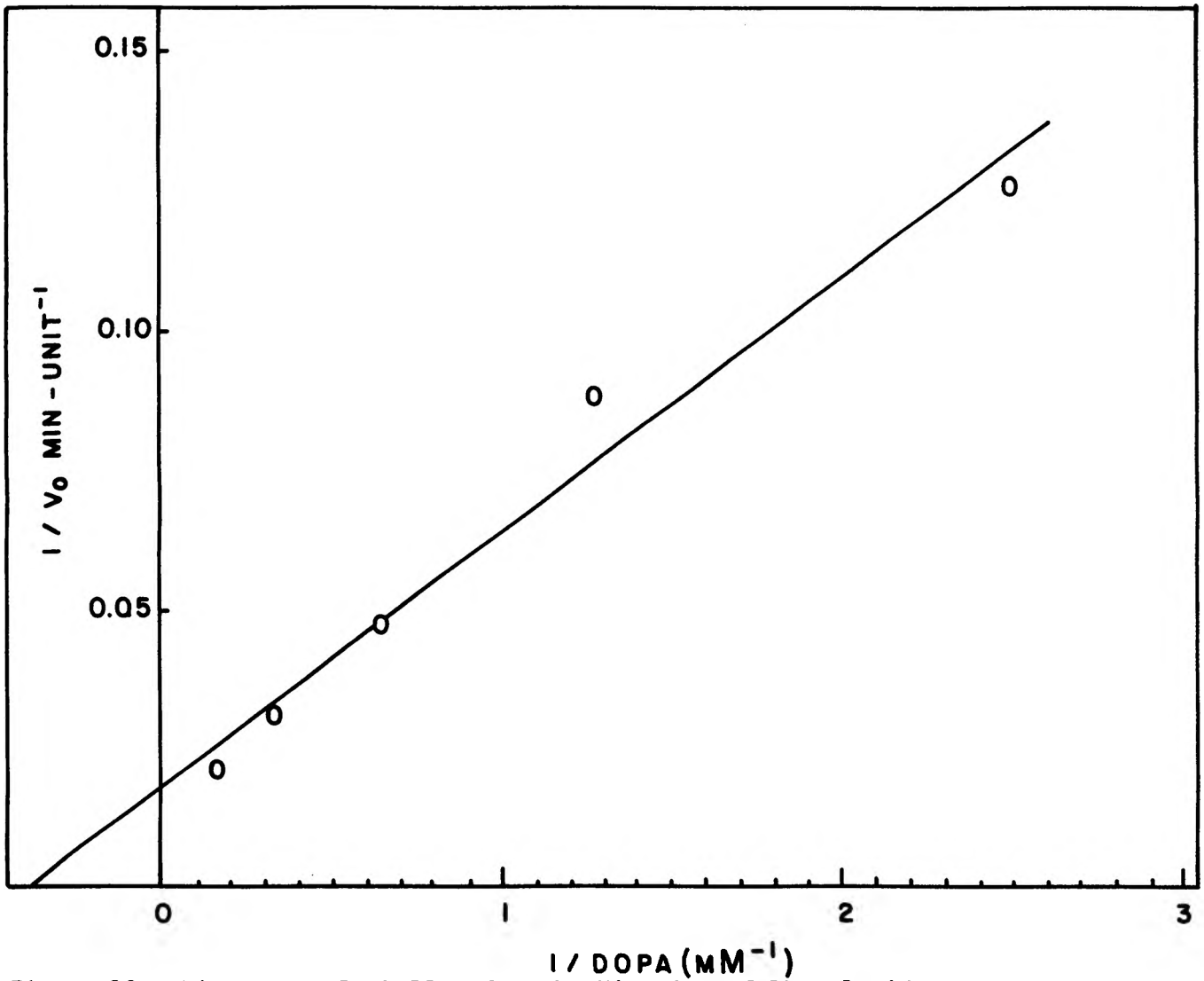


Figure 12. Lineweaver-Burk Plot for the Kinetics of Phenoloxidase

TABLE VII

MICHAELIS-MENTEN CONSTANTS (K_m) FOR VARIOUS PHENOLOXIDASES USING DOPA AS SUBSTRATE

Source Organism	Location	PO type	K_m (mM)
Aspergillus niger	intracellular	tyrosinase	0.25
Hamster melanoma	intracellular	tyrosinase	0.50
Pseudomonas melanogenum	intracellular	tyrosinase	0.99
Schizophyllum commune	intracellular	laccase	4.25
Coprinus macrorhizus var. *microsporus*	extracellular	laccase	2.33

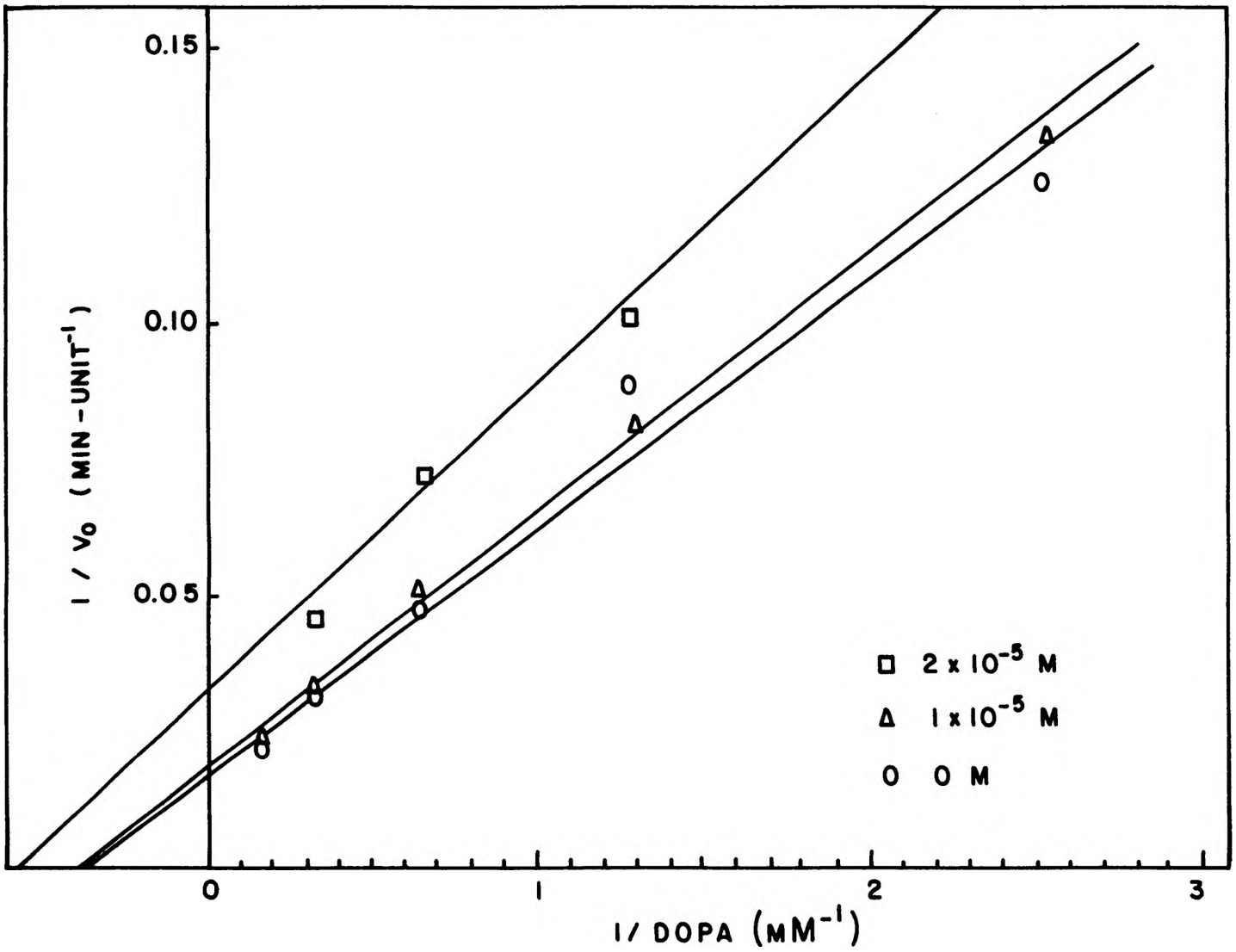


Figure 13. Lineweaver-Burk Plot for the Kinetics of Phenoloxidase Inhibition by Cysteine

The purified phenoloxidase preparations were examined by disc gel electrophoresis [39]. Four active bands were detected, two which were significantly more mobile. Nine protein bands were detected and those which were in highest concentration, as determined by the band intensities, corresponded to the active bands. This pattern of active bands corresponds well to the A. niger tyrosinase [18]. Four active bands were also detected, two of which moved significantly faster in the gel matrix.

The phenoloxidase specific activity of crude cell-free extracts of S. commune 10-day-old fertile mycelia was generally 5-6 units/mg [26]. In homokaryotic mycelia, the phenoloxidase activity was barely detectable. On aging, after 8-days growth, the specific activity increased and remained at approximately 1 unit/mg. The specific activity of 8-day-old wild homokaryotic C. macrorhizus microsporus is approximately 3 units/mg. In the mutant homokaryotic Coprinus, specific activities of 19 and 50 units/mg. were obtained in the cell-free crude medium, significantly higher than any of the S. commune preparations.

These results support the conclusion that the characteristics of the extracellular phenoloxidase isolated from C. macrorhizus microsporus (5074) are similar to other phenoloxidases. The Coprinus phenoloxidase was not investigated as to the presence of ortho-hydroxylase activity, and therefore a description of the enzyme as a tyrosinase or laccase cannot be made. Certain characteristics suggest a tyrosinase. A solution of the purified

enzyme is yellow in contrast to the blue color of laccases [18]. Also, the disc gel electrophoretic pattern of tyrosinase from A. niger is comparable to Coprinus phenoloxidase. An investigation of the enzyme's substrate specificity would provide data necessary in making suitable classification.

A significant difference in phenoloxidase activity is observed in the mutant (5074) and wild-type (5377) strains of C. macrorhizus microsporus (Fig. 6). The concomitant formation of purple pigment, hydroxylagopodin B, may be related to the increased enzyme activity, specifically by hydroxylation of the logical precursor, lagopodin B. Lagopodin B is a yellow pigment which has been isolated from the wild-type (5377) and detected by thin-layer chromatography in the mutant C. macrorhizus microsporus (5074).

To my knowledge the enzymatic hydroxylation of quinones is not known. Considering the phenolic nature of the various substrates oxidized by phenoloxidase, it is plausible that hydroxylation occurs on the reduced quinone, i.e. the quinol. The oxidation of quinols readily occurs by simple aeration of the solution. The conversion of lagopodin B to hydroxylagopodin B may proceed by a route as given in Fig. 14. Other evidence which supports this conclusion includes the fact that the quinol of coprinin is present in cultures of C. radians during early stages of growth. The formation of lagopodin B from lagopodin A may occur similarly.

A recent paper by Bu'Lock and Darbyshire [44] suggested that lagopodin B found in the cultures of monokaryotic and dikaryotic

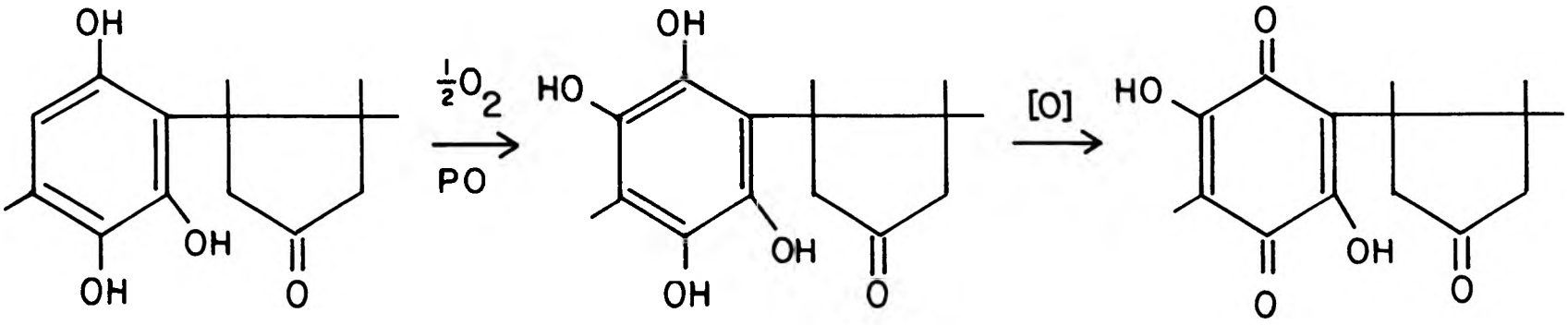


Figure 14. Postulated Scheme for the Formation of Hydroxylagopodin B

Coprinus cinereus is the result of a non-enzymic conversion from lagopodin A, especially at neutral or slightly alkaline pH. They also encountered the dimeric quinone lagopodin C and hydroxy-lagopodin B and suggested that these may be an artefact in their cultures and in the cultures of C. macrorhizus microsporus isolated by Bottom and Siehr [42]. Hydroxylagopodin B is specifically produced by a mutant strain (5074, ATCC 34960) of C. macrorhizus microsporus. Cultures of the compatible wild-type strain (5377) do not produce hydroxylagopodin B even in two-month-old cultures. In addition, the dikaryon (5377 x 5074) of C. macrorhizus microsporus does not produce the purple pigment, hydroxylagopodin B. The pH of the medium of wild-type (yellow) and mutant (purple) cultures after 12-days growth were nearly equivalent at 6.6 and 6.7 respectively.

A solution of 2,5-dimethyl benzoquinone in dilute NaOH solution stirred vigorously was yellow initially but became very dark brown after 5 minutes. On acidification, the dark solution became yellow (as with the natural lagopodins) and nearly all of the yellow pigment was extracted into ethyl acetate. The ethyl acetate phase was then extracted with 0.1M $NaHCO_3$. The fourth bicarbonate extract was purple in color and probably represents a solution of dimethylhydroxybenzoquinone.

An attempt was made to carry out the conversion of lagopodin B to hydroxylagopodin B with the purified phenoloxidase from mutant C. macrorhizus microsporus and a commercial tyrosinase (Sigma).

The experiment was conducted on a white porcelein spot plate on which the production of the purple hydroxylagopodin should be obvious. A solution of lagopodin B was added to the spot plate wells. The quinol of lagopodin B was formed in some wells by reduction with ascorbic acid and sodium borohydride. Isolated Coprinus phenol-oxidase and commercial tyrosinase were dissolved in 0.05M phosphate buffer, pH 7.2. The enzymes were active as phenoloxidases since the red-colored product of the reaction, dopachrome, was formed using dopa as substrate. A portion of each enzyme solution was added to a set of three reaction solutions: sodium borohydride-reduced, ascorbate-reduced and untreated lagopodin B. An appropriate control was used in each case. No changes in color were noted in any of the reactions, including quinol solutions which were re-oxidized by aeration with O_2. Although the results of this crude experiment did not support the hypothesis, the evidence remains strong that hydroxy-lagopodin B is a product of the metabolism of C. macrorhizus microsporus (5074) and not simply an artefact.

V. CONCLUSION

The lagopodins are secondary metabolites originally isolated from the culture medium of Coprinus lagopus (=cinereus) and possess antibiotic activity against gram-positive bacteria [8]. These compounds are substituted benzoquinones and are responsible for some of the yellow pigmentation of the culture medium. Lagopodin B was isolated from the culture medium of a wild-type strain of Coprinus macrorhizus microsporus (5377). The culture medium of a mutant strain of C. macrorhizus microsporus (5074, ATCC 34960) turns purple after 8 days growth and contains a series of pigments. One of the purple pigments was identified as hydroxylagopodin B and differs from the structure of lagopodin B by the substitution of a hydroxy group on the benzoquinone nucleus. Lagopodin B was also present in the culture medium of the mutant strain as indicated by thin-layer chromatography. The mutant strain specifically produces hydroxylagopodin B and its production is probably related to the mutation. This is the first report of this compound [42] although Bollinger [8] attempted to synthesize hydroxylagopodin B by hydrolysis of the aniline derivatives (X and XI, Fig. 5) of lagopodin B.

In the culture medium of the mutant strain, the phenoloxidase activity increases significantly after 8 days growth which is concomitant with purple pigment production. The phenoloxidase activity in the culture medium of the wild-type strain remains constant during the same growth period. The kinetic characteristics

of the isolated and purified phenoloxidase compares well with those of phenoloxidases from other sources. The production of hydroxylagopodin B and the significant increase in phenoloxidase activity are probably not directly related, since attempts to hydroxylate lagopodin B as the quinone and quinol with the purified phenoloxidase failed. The suggestion that hydroxylagopodin B may be an artefact [44], the result of pH-dependent non-enzymic reactions, is not supported by our results with C. macrorhizus microsporus (5074). The pH of wild-type and mutant strain medium is nearly identical after 10 days growth. The formation of purple pigment and increased phenoloxidase activity are probably the consequence of the mutation, which was characterized as a single-gene mutation [3]. From the available data, it is nearly impossible to elucidate the biochemical nature of this mutation.

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APPENDIX B

ABBREVIATIONS

ATCC - American Type Culture Collection

dopa - 3,4-dihydroxyphenylalanine

dopachrome - 2-carboxy-2,3-dihydroindole-5,6-quinone

dopaquinone - phenylalanine-3,4-quinone

leukodopachrome - 2-carboxy-2,3-dihydro-5,6-dihydroxyindole

ms - mass spectrum

nmr - nuclear magnetic resonance

tlc - thin-layer chromatography

PART II

The Cell Wall Composition and Structure of *Coprinus macrorhizus microsporus*

Structure of an Alkali-Soluble Polysaccharide from the Hyphal Wall of the Basidiomycete, Coprinus macrorhizus var. microsporus

CAREY B. BOTTOM and DONALD J. SIEHR

Department of Chemistry
University of Missouri-Rolla
Rolla, MO 65401 (U.S.A.)

ABSTRACT

A unique, alkali-soluble polysaccharide has been isolated from the cell walls of the basidiomycete, Coprinus macrorhizus microsporus. The polysaccharide, which is primarily glucan in nature, contains a large proportion of α-(1→4)-linked D-glucose residues and a smaller amount of β-(1→3),(1→6) linkages as suggested by methylation, partial acid hydrolysis, periodate, and enzymic studies. Hydrolysis of the methylated polysaccharide gave equimolar amounts of 2,4-di- and 2,3-di-O-methyl-D-glucose; no 2,6-di-O-methyl-D-glucose was identified indicating the absence of branch points joined through O-1, O-3 and O-4. The isolation and identification of 2-O-α-glucopyranosyl erythritol from the periodate oxidation of the polysaccharide suggests that segments of the α-(1→4)-linked D-glucose residues are joined by single (1→3)-linkages. An extracellular enzyme preparation from Sporotrichum dimorphosporum (QM 806) containing both β-(1→3) and α-(1→4) glucanohydrolase activity released 76% of the reducing groups from the polysaccharide. The polysaccharide also contains minor amounts of xylose, mannose, glucosamine and amino acids.

INTRODUCTION

As is true with fungi, the mycelial cell wall of Basidiomycetes is primarily carbohydrate. In cases where it has been studied, the cell wall has been shown to be composed of two or more distinct polysaccharides differing in either monosaccharides or type of glycosidic linkage or both.

The structure of the alkali-soluble (S-glucan) and alkali-resistant (R-glucan) fractions derived from the cell wall of Schizophyllum commune have been extensively investigated[1,2]. S-glucan was shown to be primarily a (1→3)-α-D-glucan with a small number of α-D-(1→6) linkages[1]. This fraction also contains xylose and mannose. These may be present as distinct polymers but this has not been established. The R-glucan is a (1→3)-β,(1→6)-β-D-glucan containing chitin and protein. In Polyporous tumulosus cell walls[3], the alkali-soluble fraction contains two polysaccharides, a (1→3)-α-D-glucan and a xylomannan. Information on the cell wall of the common mushroom, Agaricus bisporus is largely qualitative although the alkali-soluble fraction was assumed to be an α-D-glucan[4]. The structure of an alkali-soluble polysaccharide from the cell walls of Sporotrichum dimorphosporum (Basidiomycete QM 806) has been partially characterized[5]. The polysaccharide is a β-D-glucan containing a mixture of (1→3), (1→6) and (1→4)-β-D-linked glucopyranosyl residues.

Recently, Schaefer[6] has reported on the structure of an alkali-soluble polysaccharide from the cell walls of the basidiomycete, Coprinus lagopus (=cinereus). Unlike, S. commune, this polysaccharide is a β-D-glucan primarily (1→3)-linked with (1→6) branch points.

Marchant[7] examined the wall composition of monokaryotic and dikaryotic mycelia in Coprinus cinereus. Dikaryotic cell walls contained no alkali-soluble fraction and significantly less chitin which was replaced by hexose. The alkali-soluble polysaccharide from monokaryotic cell walls was assumed to be a β-(1→3)-β-(1→6)-D-glucan as reported by Schaefer.

Developmental aspects of Coprinus macrorhizus microsporus growth have been documented[8-15], however the structure of its cell wall polysaccharides is unknown. This work reports on the structure of an unique alkali-soluble polysaccharide from the hyphal wall of this organism.

RESULTS

The cell walls of Coprinus macrorhizus microsporus (5377) were prepared by treatment using a combination of techniques: freeze-thawing, homogenization, and sonic oscillation. Sodium dodecyl sulfate and was added during treatment by sonic oscillation to remove cytoplasm and non-wall protein[16]. Extraction of the lipid-free walls with superheated water was carried out to remove any intracellular glycogen and soluble protein[17] that may have adhered to the cell wall preparation. Fractionation with 1M alkali carried out at three temperatures, 0°, 21°, and 60°C for 1.5, 18 and 1.5 hours respectively, gave three alkali-soluble fractions (I, II, III) representing 26% of the original wall weight. Fraction II, representing 12% of the original wall weight, is the subject of this communication. Fractions I and III have not been characterized, although they gave glucose on

hydrolysis when examined by paper chromatography. It is assumed that these fractions have similar constitution and structure to fraction II. The purified polysaccharide was only partially soluble in water.

Electrophoresis on paper of fraction II gave a single spot which migrated 1.2 cm toward the cathode. Under the same conditions glucose migrated 4.0 cm toward the anode. The complete acid hydrolysate of fraction II contained glucose and mannose plus a trace reducing spot that also gave a positive ninhydrin reaction as determined by paper chromatography. The ninhydrin positive material was glucosamine. The hydrolysate contained other ninhydrin positive spots which were shown to be amino acids. The products from the complete acid hydrolysate were also separated by glc as the BBA ester derivative[18] of the corresponding alditols. Mannose was detected in the amount of 6% relative to glucose by chromatogram area assuming equivalent molar response factors[18]. A trace amount of glucosaminitol-BBA was also noted.

The products from the partial acid hydrolysis[19] was a mixture of oligosaccharides, three disaccharides, glucose, and a spot corresponding to xylose as shown by paper chromatography. The disaccharides were laminaribiose, maltose and gentiobiose which were identified by comparison to authentic standards using paper chromatography in two solvent systems (A and B). Lichenan, which contains β-(1→3) [30%] and β-(1→4) [70%] linkages[20], was hydrolyzed under identical conditions and provided a standard for laminaribiose. Maltose and cellobiose are not readily resolved in most solvent systems, however solvent system B gave sufficient separation to identify the two disaccharides. No glucosamine was detected in the partial acid hydrolysate.

The optical rotation of the polysaccharide, $[\alpha]_D^{25}$ = +85° (ca. 0.7% in 1M NaOH), suggested that fraction II was a glucan that was primarily α-linked. The optical rotation was different from that of other alkali-soluble glucans from Basidiomycetes. These have either specific optical rotation on the order of +200°[1-3] if α-linked or 0°[6] if β-linked. The infrared spectrum of fraction II gave a pattern of absorptions at 920, 885 (shoulder), 835 and 748 cm^{-1}. The S-glucan from S. commune had absorptions at 923, 850, 830 and 775 cm^{-1} and lichenan gave a single large peak at 888 cm^{-1}. Barker[21], et al. have suggested that α-linked and β-linked polysaccharides exhibit absorptions at 844 ± 8 cm^{-1} and 891 ± 7 cm^{-1} respectively. The primary α-nature of fraction II glucan is confirmed by absorption at 835 cm^{-1}; a shoulder at 885 cm^{-1} indicates some β-linkages may be present. Fraction II does not give a color with iodine-potassium iodide solution.

The alkali-soluble polysaccharide was hydrolyzed enzymically with a partially purified enzyme preparation obtained from the culture filtrate of S. dimorphosporum (QM 806). These results along with activities against various other substrates are reported in Table I.

Sporotrichum dimorphosporum (QM 806) is an excellent source of an exo-β-(1→3) glucanase. However, the partially purified enzyme preparation contains significant amounts of α-(1→4) glucanohydrolase activity. The exact specificity of the α-(1→4) glucanohydrolase is not known. With amylose and glycogen the amount of glucose released was greater than the reducing sugar liberated (Table I); this indicated partial thermal stability of the enzyme to inactivation by

boiling 5 minutes similar to that observed with the α-(1→4) glucano-hydrolases, beta-amylase and pullulanase[22]. No β-(1→6) activity and only trace amounts of cellulolytic activity were detected. The enzymic hydrolysate of C. macrorhizus microsporus alkali-soluble polysaccharide was concentrated, deionized and examined by paper chromatography. Glucose and gentiobiose were the only sugars identified. Unidentified higher oligosaccharides were also present.

The glucan was methylated by the method of Hakomori[23] and found to be completely methylated as suggested by the absence of infrared absorption in the region 3400-3500 cm^{-1} and by the detection of only a trace amount of mono-O-methyl glucose ether. The permethylated glucan gave on hydrolysis five methyl sugars identified by silica gel thin-layer chromatography as 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, 2,3,6-tri- and 2,3-di-O-methyl-D-glucose. Their identity was confirmed by gas-liquid chromatography as the alditol acetates[24]. The two di-O-methyl ethers of glucose, 2,3-di-O-methyl- and 2,4-di-O-methyl-, which apparently are not separated by thin-layer chromatography in the solvent system employed, are separated by glc. Retention times of the unknowns matched those of authentic standards. Mole percentages of the methyl sugars obtained on hydrolysis are reported in Table II. Two minor peaks were obtained on methylation analysis. Unknown methyl sugars 1 and 2 are tentatively identified as 2,3,4-tri-O-methyl-D-xylose and 2,6-di-O-methyl-D-mannose respectively based on their relative retention time and comparison to literature values[24].

The alkali-soluble glucan consumed 0.95 moles of periodate and released 0.19 moles of formic acid per mole of anhydroglucose. These

results are in nearly perfect agreement with the methylation results. The expected periodate consumed based on methylation would be 0.97 moles per mole of anhydroglucose. The expected formic acid released would be 0.22 moles. The oxidized polysaccharide was reduced with sodium borohydride and the resultant polyalcohol subjected to mild acid hydrolysis at room temperature. The hydrolysate was concentrated in vacuo at 40°C, deionized, and examined by cellulose thin-layer chromatography. Erythritol and glycerol were detected and identified by co-chromatography on tlc plates with standards. The identity of these products were confirmed by gas-liquid chromatography of their acetates on a 3% ECNSS-M column. Retention times of the unknows were identical to those of standards acetylated by the same procedure. Quantitation by gas-liquid chromatography indicated a 2.1:1 mole ratio of erythritol to glycerol. This result is also in agreement with methylation results, since erythritol is derived from glucose residues linked either through 1 and 4 or 1, 4 and 6 whereas glycerol would be expected from end-groups, glucose residues linked through either 1 and 6 or 1, 2 and 6. The sugar alcohol acetates were separated by silica gel thin-layer chromatography and detected by the ferric hydroxamate reaction. The presence of erythritol tetra-acetate (R_f 0.58), glycerol tri-acetate (R_f 0.66) and an unknown acetate (R_f 0.23) was established. Since the glucan contains (1→3), (1→4), and (1→6) linkages, it was suspected that the unidentified product may be 2-O-glucopyranosyl erythritol or 3-O-glucopyranosyl glycerol arising from (1→3) linked glucose residues which are flanked by (1→4) or (1→6) linkages respectively. These glycosides do not react with reducing substances. The proportion of the unknown product as indicated by the

intensities of the acetate spots on silica gel thin-layer chromatograms suggested the former as a logical choice. The 2-O-α-glucopyranosyl erythritol was synthesized from maltose by lead tetraacetate oxidation followed by reduction with sodium borohydride[25]. The configuration of the linkage was supported by the previous results of the enzymic and partial acid hydrolysis. The unknown product was identified as 2-O-α-D-glucopyranosyl erythritol as suggested by equivalent mobilities of the acetate derivatives on silica gel thin-layer chromatograms.

Paper chromatography of the polysaccharide acid hydrolysates indicated ninhydrin-positive components other than the amino sugar glucosamine. The amino acid composition of the polysaccharide was determined and these results are reported in Table III. The analysis shows relatively high proportions (>10%, w/w) of alanine, glycine, aspartic acid and glutamic acid. Aspartic and glutamic acid may form linkages between carbohydrate and protein. Total protein content based on the amino acid analysis was 1.2% of the dry weight.

DISCUSSION

The alkali-soluble polysaccharide from the cell walls of Coprinus macrorhizus microsporus differs from those reported for other Basidiomycetes[1-5] and a generically related species[6]. Methylation studies revealed the presence of (1→4)-, (1→3)-, (1→6)-linked D-glucopyranosyl residues with both (1→4)- and (1→3)-linked D-glucopyranosyl residues having branches at O-6. The presence of (1→4) linkages in the alkali-soluble fraction is substantiated by the detection of erythritol on

hydrolysis of the reduced, periodate-oxidized glucan, hydrolysis by an α-(1→4) glucanohydrolase obtained from Sporotrichum dimorphosporum, and the identification of maltose in the partial acid hydrolyate.

The occurrence of (1→4) linkages in Basidiomycetes is unusual and has been reported in only one other member[5], S. dimorphosporum. In S. dimorphosporum, however, the configuration of the (1→4) linkage was assumed to be β based on the specific optical rotation of the polysaccharide. No partial acid hydrolysis was carried out. Enzymic hydrolysis of the S. dimorphosporum polysaccharide with an exo-β-(1→3) glucanase isolated from the same organism, released 38% of the reducing groups. Further analysis of the enzyme resistant fraction by methylation, suggested that the β-(1→3) glucanase hydrolyzes (1→4) linkages as well as β-(1→3) linkages. The enzyme was not able to hydrolyze the β-(1→3),(1→6) branch points. No hydrolytic activity was detected against the substrates cellulose or carboxymethylcellulose, but the preparation was not assayed against α-D-glucans. Although the exo-β-(1→3) glucanase used in the work reported here was not purified to the same extent, it is possible that the preparation of Bush and Horisberger[5] contained α-(1→4) hydrolase activity, and they may have observed the hydrolysis of α-(1→4) linkages.

The major alkali-soluble polysaccharide, fraction II, was isolated from rigorously prepared cell walls by alkali-extraction and its homogeneity was indicated on paper electrophoretigrams. The specific optical rotation, $[\alpha]_D^{25°}$ = +85°, implied an α-linked D-glucan, however this intermediate value, as compared to other glucans of only one type of linkage-configuration supports the conclusion that the

polysaccharide is a mixed-configurational glucan. The infrared spectrum was in agreement with this since a shoulder was observed at 885 cm^{-1} characteristic of a β-D-glucan, in addition to a significant absorption at 835 cm^{-1} typical of α-linked glucans.

The glucan was extensively hydrolyzed with an enzyme preparation from S. dimorphosporum. Seventy-six percent of the reducing groups and 65% of the glucose (Table I) was released. The large amount of hydrolysis can only be explained by the action of both α-(1→4) and β-(1→3) hydrolase activities present in the enzyme preparation (Table I). Gentiobiose was the only disaccharide identified in the enzyme hydrolysate. Small amounts of higher oligosaccharides were also present and these may be a homologous series of β-(1→6) linked D-glucose residues as was found in the R-glucan of S. commune[2].

The identification of maltose, laminaribiose, and gentiobiose in the partial acid hydrolysate substantiated the results of methylation analysis and further characterizes the linkages as α-(1→4), β-(1→3), β-(1→6). It is possible that some of the (1→6) linkages may be α, although we have no direct proof that this is the case.

The polysaccharide appears to be a highly-branched glucan with branch points at O-6 on both (1→4)- and (1→3)-linked residues. The amount of end groups is nearly equal to the sum of branch points, hence little or no crosslinking occurs between the glucan chains. Since no 2,6-di-O-methyl-D-glucose was detected on methylation analysis and crosslinking was ruled out, it was apparent that the attachment of (1→4) linkages was via an occasional (1→3) link as borne out by the identification of 2-O-α-glucopyranosyl erythritol. Branching

at O-6 occurs in one glucose residue for every eight (1→4) linked residues. A branch point at position 6 occurs at one glucose residue for three (1→3) linked residues. Preliminary methylation studies on the glucan of the alkali-insoluble fraction, indicate a very similar structure to that of the alkali-soluble polysaccharide (fraction II).

Xylose and mannose are present in the alkali-soluble polysaccharide from C. macrorhizus microsporus. Only glucose was observed on a paper chromatogram of the hydrochloric acid hydrolysate. Glc separation of the BBA ester derivatives of the hydrolysate gave an indication of mannose as well as glucose. The small amount of mannose was probably masked by glucose on the paper chromatogram since these epimers are not well-resolved in many solvent systems. Xylose was present in the partial acid hydrolysate as shown by paper chromatography, but was not present in the hydrochloric acid hydrolysate. It was assumed that this stronger hydrolytic condition resulted in nearly complete destruction of the xylose. This has also been observed with the xylose in the cell walls of P. tumulosus[3]. In P. tumulosus the xylose was derived from a water-insoluble xylomannan separated and purified as a copper complex, from the alkali-soluble fraction of the cell walls[3]. The structural characteristics of the xylomannan were similar to a xylomannan isolated from the hyphal wall of Armillaria mellea, a Basidiomycete[26]. However the Armillaria xylomannan does not form a copper complex and is soluble in water. The alkali-soluble glucan from S. commune also contains mannose and xylose[1,2]. Siehr[1] was unsuccessful in precipitating a xylomannan as a copper complex from the alkali-soluble fraction of S. commune.

The xylomannan of P. tumulosus cell walls is an α-(1→3)-linked mannan to which xylose residues are attached[3]. Permethylation followed by hydrolysis gave 2,3,4,6-tetra-O-methyl mannose, 2,3,4-tri-O-methyl xylose, 2,4,6-tri-O-methyl mannose and a dimethyl mannose tentatively characterized as 2,6-dimethyl mannose. The trimethyl xylose, trimethyl mannose and dimethyl mannose were present in approximately equal proportions. Methylation of the alkali-soluble polysaccharide of C. macrorhizus microsporus cell walls gave equimolar amounts of methyl sugars tentatively characterized as 2,3,4-tri-O-methyl xylose and 2,6-di-O-methyl mannose by relative retention times[24]. Other methylmannoses may have eluted with methylglucose analogs, since it is known that they have comparable retention times. Xylose and mannose has been reported in many fungal species especially Basidiomycetes[3]. Xylose and mannose occur in the alkali-soluble cell wall fraction of C. macrorhizus microsporus perhaps as a xylomannan, and the structure may be similar to the xylomannan of P. tumulosus. These xylomannans may have common structural roles in related species of filamentous fungi[3].

Many fungal cell wall preparations contain protein and it is now clear that it is a structural component[27]. The alkali-soluble fraction of C. macrorhizus microsporus cell walls contain protein, as other alkali-soluble cell wall fractions of Basidiomycetes. Siehr[1] reported amino acids in the S-glucan of S. commune although no specific identification was carried out. Sietsma and Wessels[2] reported 2.2% (wt.) amino acids in their S-glucan preparation quantitated by the ninhydrin assay. The amino acids were not identified. In

addition, they report a small amount of glucosamine (0.2% by wt.) in this fraction, which probably occurs as N-acetylglucosamine in the original polysaccharide. A small amount of glucosamine was detected in the alkali-soluble fraction of C. macrorhizus microsporus as evidenced by paper chromatography and glc of the BBA derivative. Protein which occurs in our preparation contains a high proportion of acidic amino acids (Table II). The carbohydrate and protein of yeast mannan are covalently-linked primarily through asparagine by way of di-N-acetylchitobiose and through O-glycosidic bonds via hydroxyamino acids[28]. These are characteristic linkages of glycoproteins. Although the information available is scanty, it is reasonable to speculate that the carbohydrate moeity of the alkali-soluble cell wall fraction of C. macrorhizus microsporus may be linked covalently to asparagine or glutamine residues in the protein through N-acetyl-glucosamine or its dimer.

The cell wall structure of fungi which are closely related in terms of morphology would not be expected to differ drastically[29]. Structurally, the alkali-soluble polysaccharide from C. macrorhizus microsporus cell walls is substantially different from those reported in other Basidiomycetes. These results suggest a diversity in the cell wall structure of related fungi which may be species or perhaps strain specific. Generalizations on the structural features of cell walls from related organisms should be closely scrutinized.

EXPERIMENTAL

General methods. — Total carbohydrate was determined by the anthrone method[30], reducing sugars by the dinitrosalicylic method[31], and D-glucose specifically by the D-glucose oxidase method as modified by Lloyd and Whelan[32]. Chromatography on Whatman No. 1 paper was accomplished in the descending manner using ethyl acetate-pyridine-water (12:5:4, v./v., solvent A) or butanol-pyridine-water (2:1:1, v./v., solvent B)[33]. Reducing sugars were visualized with alkaline silver nitrate[33]. Glucosamine was detected with the ninhydrin reagent[33]. Sugar alcohols were separated by cellulose F (E. Merck) thin-layer chromatography using n-propanol-ethyl acetate-water (7:1:2, v./v.)[34]. Detection was with alkaline silver nitrate[34]. Sugar and sugar alcohol acetates were separated by thin-layer chromatography using silica gel G (E. Merck) plates developed with 4% methanol in benzene (v./v.)[35]. The acetates were detected by the ferric hydroxamate reaction[35]. Methyl sugars obtained on hydrolysis of the permethylated polysaccharide were separated on silica gel G (E. Merck) plates using methylene chloride-methanol (9:1, v./v.) for development[36]. Infrared spectra of polysaccharides were measured in a potassium bromide matrix using a Perkin-Elmer Model 180 IR spectrophotometer at 40°C. The optical rotation was measured in 1 M sodium hydroxide with a Kern polarimeter. High-voltage electrophoresis was carried out on Whatman No. 1 paper for 1 hr using 0.01 M borate buffer, pH 9.85. Visualization was with periodate-benzidine[33] or alkaline permanganate[37].

Organisms. — Coprinus macrorhizus var. microsporus (5377) was kindly provided by Dr. Philip G. Miles (Dept. of Biology, SUNY, Buffalo, NY) who originally obtained the culture through the courtesy of Dr. Tsuneo Takemaru (Dept. of Biology, Okayama University, Okayama, Japan). Sporotrichum dimorphosporum (Basidiomycete QM 806) was obtained from the culture collection of the University of Massachusetts, formerly the collection of the US Army Natick Laboratories, Natick, MA.

Media and cultural conditions. — C. macrorhizus microsporus was cultured on complete medium[38] supplemented with 0.05% (w./v.) yeast extract. The inoculum was prepared by maceration of mycelia removed from a 10-day-old agar plate maintained at 25°C. The suspension was transferred to 1 l. liquid medium contained in 2.8 l. Fernbach flasks and incubated with shaking (90 rotations/min.) for 11 days. S. dimorphosporum was cultured on liquid medium as described by Peterson and Kirkwood[39].

Preparation of cell walls. — The cells (4x1 l. cultures) were recovered by vacuum filtration on two layers of cheesecloth, washed extensively with distilled water, and immediately frozen. Thawed cells (66 g. wet wt.) were washed with distilled water by vacuum filtration and suspended in 400 ml. 0.8% sodium chloride (w./v.). These were homogenized in a Waring blender at Hi speed for one minute. The homogenate was centrifuged at 16000 g (15 min.).

The recovered residue was washed with distilled water (2X) and the mycelial fragments collected by centrifugation at 10000 g (15 min.). Collected mycelial fragments were frozen and stored at 20°C.

Thawed fragments were suspended in 1% sodium dodecyl sulfate (w./v.) and sonicated at maximum output for two minutes at 30 sec intervals. The cell walls were collected by centrifugation at 10000 g (30 min.) and washed 2X with distilled water by centrifugation. The cell walls were then suspended in distilled water, dispersed in a Waring blender, and sonicated at maximum output for 1 min. at 30 sec. intervals. Recovery was effected by centrifugation at 10000 g (15 min.) and the residue washed with distilled water (2X) by centrifugation. Sonication (1 min. at 30 sec. intervals) of the residue was repeated in the presence of 0.8% sodium chloride, followed by centrifugation at 8000 g (15 min.). This residue was frozen, thawed, suspended in water and dispersed in a Waring blender at Hi speed. Cell walls were recovered by centrifugation at 10000 g (15 min.), washed with water, collected by centrifugation at 8000g (15 min.), followed by a second wash and collection by centrifugation at 5000 g (15 min.). Examination of the final residue using a light microscope and methylene blue stain indicated nearly complete cell breakage and no cytoplasmic contamination. The cell walls were lyophilized, recovered (yield 4.50 g) and stored at 20°C. Freeze-dried cell walls were extracted on a Soxhlet with chloroform-methanol (2:1, v/v) for 66 hrs. Lipid-free cell walls were dried to constant weight in a 80°C oven (yield 4.05 g).

Cell wall fractionation. — Lipid-free cell walls (3.85 g) were autoclaved in distilled water (150 ml) for 1 hr at 122°C, cooled, and recovered by centrifugation at 10000 g (15 min). The cell walls were washed 2X by centrifugation, dialyzed against distilled water for 2 days at 4°C. Total carbohydrate (208 mg) and free glucose (0 mg)

extracted into hot water were measured by the anthrone and glucose oxidase methods respectively. The carbohydrate extracted into hot water was not precipitable with cold ethanol. The cell walls were then extracted with 1 M sodium hydroxide (150 ml) at 0°C with stirring under nitrogen for 1.5 hrs. The suspension was centrifuged at 10000 g (15 min) and the residue washed with distilled water 2X by centrifugation. The combined supernatants were neutralized with glacial acetic acid and the polysaccharide precipitated with cold ethanol (2 vol). The 0°C-alkali-soluble polysaccharide (fraction I) was recovered by centrifugation and washed twice with cold ethanol. The recovered polysaccharide was again suspended in water, dialysed 2 days at 4°C against several changes of distilled water and finally freeze-dried (yield 132 mg). No further analysis was carried out with this fraction. The residue (fraction I-R), after alkali-extraction, was suspended in water, dispersed in a Waring blender at Hi speed, neutralized with glacial acetic acid, dialysed, and freeze-dried (yield 2.74 g). Fraction I-R (2.56 g) was extracted with 1 M sodium hydroxide (150 ml.) at 21°C with stirring under nitrogen for 18 hrs. The polysaccharide (fraction II, yield 419 mg) was recovered and treated as described for fraction I. The insoluble residue, (fraction II-R, yield 1.62 g) was carried through another alkali extraction with 1 M sodium hydroxide at 60°C with stirring under nitrogen for 1.5 hrs. The recovered polysaccharide (fraction III, yield 83 mg) was not further characterized.

Polysaccharide hydrolysis and analysis. — Polysaccharide samples were hydrolyzed in 2M HCl at 100°C for 2 hrs in a sealed ampoule. HCl

was removed by evaporation in vacuo at 40°C with repeated additions of water. The hydrolysate was stored over KOH in vacuo to remove residual traces of HCl. Hydrolysates were examined by paper and gas-liquid chromatography. The butane boronic acid (BBA) ester derivatives of the reduced monosaccharides obtained by HCl hydrolysis were prepared as described by Eisenberg[18]. Separation was on a 3% OV-17 (Chromosorb WHP, 80/100 mesh) 2m x 2mm i.d. glass column as recommended. The column temperature was 220°C; the inlet and transfer temperatures were 240°C. Helium was used as carrier gas at a flow rate of 40 mls/min. Peak areas were measured by electronic integration.

Partial hydrolysis was carried out according to the method of Johnston[19]. A 20 mg sample of polysaccharide was suspended in 0.5 ml 88% formic acid, heated at 100°C for 10 min, followed by the addition of 4 mls 0.4 N sulfuric acid and continued heating for 1 hr. Neutralization was accomplished with barium carbonate and the supernatant concentrated under reduced pressure. The hydrolysate was examined by paper chromatography using solvent systems A and B.

Methylation analysis. — The polysaccharide was twice methylated according to the procedure of Hakomori[23] as modified by Sandford and Conrad[40]. The sample was dried in vacuo at 80°C for 4 hrs prior to methylation. Hydrolysis of the methylated glucan was carried out by the formic-sulfuric acid method[24]. An infra-red spectrum was taken as a thin film evaporated from a chloroform solution on sodium chloride plates. Five components were separated from the hydrolysate by silica gel thin-layer chromatography. These corresponded to 2,3,4,6-tetra-, 2,4,6-, 2,3,6-, 2,3,4-tri, and 2,3-di-O-methyl-D-glucose.

Identification of these methyl sugars was confirmed by glc as their alditol acetates[24]. The di-O-methyl fraction identified on thin-layer plates was a mixture of 2,4-di- and 2,3-di-O-methyl-D-glucose. Separation was accomplished on a (2m x 2mm i.d.) glass column packed with 3% ECNSS-M on Gas-Chrom Q (100/200 mesh) using helium at 40 mls/min as the carrier gas. The inlet and transfer temperatures were 250°C. Temperature programming from 140-180°C at 1°/min was used to separate all the methyl sugars. Using this technique, the resolution of the alditol acetates of 2,3,4-tri- and 2,3,6-tri-O-methyl-D-glucose was possible. Positive identification of methyl glucoses was obtained by comparison to authentic samples. Peak areas were measured by electronic integration and the relative concentration of methyl sugars calculated assuming the molar response factors are equivalent for all derivatives[24].

Periodate oxidation and Smith degradation. — Oxidation of the glucan (100 mg) was carried out in 25 mM sodium metaperiodate (100 mls) in the dark at 4°C. The consumption of periodate was determined at various time intervals by the Malaprade method as outlined by Dryhurst[41]. Formic acid production was monitored by titration with standard alkali using bromcresol purple as indicator[41]. Appropriate blanks were used in each determination. Oxidation was considered complete after 5 days. The oxidized glucan was reduced with sodium borohydride and hydrolysed under mild acidic conditions according to the method of Smith[42]. Glycerol and meso-erythritol were identified by cellulose thin-layer chromatography and as the acetate by glc on 3% ECNSS-M. Gas-liquid chromatography was carried out isothermally at

160°C with all other conditions identical to those used for methyl sugars. The acetate derivatives of the Smith products were also separated by silica gel chromatography. The 2-O-α-glucopyranosyl erythritol was synthesized according to the procedure of Charlson, et al.[25] and acetylated with acetic anhydride-pyridine mixture (1:1, v/v) and used as a standard.

Enzymic hydrolysis. — An enzyme mixture containing both β-(1→3) and α-(1→4) hydrolase activities was recovered as the 25-50% ammonium sulfate fraction. The precipitate was dissolved in 0.048 M citrate-phosphate buffer, pH 4.5, and assayed against various substrates: S-glucan from S. commune, lichenan (Koch-Light) from Cetraria islandica, laminarin (Koch-Light) from Laminaria hyperborea, pustulan from Parmelia papullosa (Calbiochem), cellulose (Solka-Floc, Brown Co.), carboxymethylcellulose (Eastman Kodak Co.), soluble starch (Fisher), amylose from potato (Sigma), glycogen from shellfish (Sigma), and dextran (Sigma). The enzyme preparation contained both exo-β-(1→3) and α-(1→4) glucanohydrolase activities. A liter of culture medium yielded 245 units[43] when assayed against laminarin. Hydrolysis of the isolated polysaccharide and various substrates was carried out in 0.048 M citrate-phosphate buffer, pH 4.5 for 12 hrs at 40°C. Toluene was added as a bacterial growth inhibitor. The amount of enzyme added in each case was 0.06 units per mg substrate. The reaction was stopped by immersion in a boiling water bath for 5 min. Insoluble substrate, if present, was removed by centrifugation prior to the determination of reducing sugar[31] and D-glucose[32].

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TABLE I

ENZYMIC HYDROLYSIS

Substrate Glucan Type	Reducing Sugar Released (%) Total by Weight	Glucose Released (%) Total by Weight
Alkali-soluble glucan (*C. macrorhizus microsporus*)	76%	65%
S-glucan (*S. commune*) [(1→3)-α,(1→6)-α-D-glucan]	4.2%	3.0%
lichenan [(1→3)-β,(1→4)-β-D-glucan]	13%	3.7%
laminarin [(1→3)-β-D-glucan]	89%	84%
pustulan [(1→6)-β-D-glucan]	0%	0%
cellulose (Solka-Floc) [(1→4)-β-D-glucan]	0.2%	0.1%
carboxymethylcellulose [(1→4)-β-D-glucan]	0.3%	0.2%
soluble starch [(1→4)-α-,(1→6)-α-D-glucan]	9.0%	9.0%
amylose [(1→4)-α-D-glucan]	53%	57%[a]
glycogen [(1→4)-α-,(1→6)-α-D-glucan]	90%	95%[a]
dextran [(1→6)-α-D-glucan]	11%	11%

[a]Enzyme was not completely inactivated by 5 min at 100°C.

TABLE II

METHYLATION ANALYSIS

O-methyl Sugar (alditol acetate)	Mode of Linkage	Mole Percentage	RRT[a]
Unknown 1		1.7%	0.65
2,3,4,6-tetra-O-methyl-D-glucose	Gp-(1→	13.5%	1.00
2,4,6-tri-O-methyl-D-glucose	→3)-Gp-(1→	13.3%	1.70
2,3,4-tri-O-methyl-D-glucose	→6)-Gp-(1→	9.1%	1.98
2,3,6-tri-O-methyl-D-glucose	→4)-Gp-(1→	47.6%	2.07
Unknown 2		1.7%	2.41
2,4-di-O-dimethyl-D-glucose	→6)-Gp-(1→ 3 ↑	6.6%	3.01
2,3-di-O-methyl-D-glucose	→6)-Gp-(1→ 4 ↑	6.6%	3.12

[a]Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

TABLE III

AMINO ACID ANALYSIS

Amino Acid	μmole amino acid/1000 μmole
aspartic acid	104.
threonine	50.1
serine	70.6
glutamic acid	121.
proline	17.6
glycine	118.
alanine	107.
valine	74.2
isoleucine	42.5
leucine	89.4
tyrosine	37.5
phenylalanine	47.1
histidine	24.0
lysine	69.1
arginine	27.9

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Structure and Composition of the Alkali-insoluble Cell Wall Fraction of *Coprinus macrorhizus* var. *microsporus*

CAREY B. BOTTOM AND DONALD J. SIEHR*

Department of Chemistry

University of Missouri-Rolla

Rolla, Missouri 65401

*Author to whom reprint requests should be directed.

ABSTRACT

The alkali-insoluble (R-) fraction from the cell walls of Coprinus macrorhizus var. microsporus is a highly-branched glucan, containing α-(1→4), β-(1→3) and β-(1→6) linkages as shown by methylation, partial acid hydrolysis and enzymic hydrolysis. The α-(1→4)-linked segments are joined by occasional β-(1→3) links as suggested by the identification of 2-O-α-glucopyranosyl erythritol in the hydrolysate of the reduced, periodate-oxidized glucan. Hydrolysis of the permethylated glucan gave nearly equimolar amounts of 2,4-di- and 2,3-di-O-methyl-D-glucose. Methylation analysis of the residue from enzymic hydrolysis, the CORE-fraction, indicated the presence of glucose residues in this fraction also linked through positions O-1, O-3, O-4 and O-6. Hydrolysates of the R-fraction contained mannose, glucosamine and amino acids in addition to glucose.

INTRODUCTION

Members of the genus Coprinus appear to be excellent models for developmental studies, for they can complete their entire life cycle on synthetic medium in a relatively short time. Each stage during development involves specific morphological changes related to precise cell-wall structures. Diverse, interrelated enzyme activities are necessary at the site of cell wall synthesis and degradation. A specific morphology is attained by regulation of these cell-wall enzymes either by alteration of activity through allosterism, isosteric inhibition, activation of enzyme precursors or the rate and timing of enzyme synthesis. It is apparent that elucidation of the cell-wall structure is vital to understanding many of the molecular events occurring during the morphogenetic sequence.

Although a considerable amount of information is available on the developmental aspects of Coprinus macrorhizus microsporus (1-8), the wall structure remains unknown. The cell wall structure of one near relative, Schizophyllum commune has been extensively studied (9,10). Bartnicki-Garcia (11) has classified the fungi into various groups based on the components of the cell wall. Although regularity in composition is observed among these groups, it is not necessarily true that the structure of cell wall polysaccharides are similar. Schaefer (12) has reported on the structure of an alkali-soluble polysaccharide from the cell walls of Coprinus lagopus (=cinereus). The alkali-soluble polysaccharide from the cell wall of C. macrorhizus microsporus (13) differs substantially from C. lagopus. Structural

studies on the alkali-insoluble cell-wall fraction of C. macrorhizus microsporus have been carried out and the results are the subject of this paper.

MATERIALS AND METHODS

Coprinus macrorhizus microsporus (5377) was obtained from Dr. Philip G. Miles (Dept. of Biology, SUNY, Buffalo, NY) who originally obtained the culture through the courtesy of Dr. Tsuneo Takemaru (Dept. of Biology, Okayama University, Okayama, Japan). Sporotrichum dimorphosporum (Basidiomycete QM 806) was obtained from the culture collection of the University of Massachusetts, formerly the collection of the U.S. Army Natick Laboratories, Natick, MA. Sporotrichum dimorphosphrum was cultured on liquid medium as described by Peterson and Kirkwood (14). C. macrorhizus microsporus was cultured on complete medium (15) supplemented with 0.05% (w/w) yeast extract and incubated with shaking for 11 days at 22°C. The mycelia were recovered by vacuum filtration on cheesecloth layers and washed extensively with distilled H_2O. Cell walls were prepared according to a method described previously (13).

Lipid-free cell walls (3.85 g) were autoclaved in distilled water (150 ml) for 1 hr at 122°C, cooled and recovered by centrifugation at 10000 g (15 min). The cell walls were washed 2X by centrifugation, exhaustively dialyzed against distilled water for 2 days at 4°C, and freeze-dried. Freeze-dried cell walls were then extracted with 1M NaOH (150 ml) at 0°C with stirring under N_2 for 1.5 hrs. The suspension was centrifuged at 10000 g (15 min) and the residue washed with distilled H_2O 2X by centrifugation. The combined supernatants were neutralized with glacial HOAc and the polysaccharide precipitated with cold ethanol (2 vol). The 0°C-alkali-soluble polysaccharide

(fraction I) was recovered by centrifugation and washed twice with cold ethanol. The polysaccharide was again suspended in water, exhaustively dialyzed against distilled water and finally freeze-dried (yield 132 mg). No further analysis was carried out with this fraction. The residue (fraction I-R), after alkali extraction, was suspended in water, dispersed in a Waring blender at Hi speed, neutralized with glacial HOAc, dialyzed, and freeze-dried (yield 2.74 g). Fraction I-R (2.56 g) was extracted with 1M NaOH (150 ml) at 22°C with stirring under nitrogen for 18 hrs. The polysaccharide (fraction II, yield 419 mg) was recovered and treated as described for fraction I. Structural characterization of fraction II has been described previously (13). The insoluble residue (fraction II-R, yield 1.62 g) was carried through another alkali extraction with 1M sodium hydroxide at 60°C with stirring under nitrogen for 1.5 hrs. The alkali-soluble polysaccharide (fraction III, yield 83 mg) from the extraction was not further characterized. The alkali-insoluble residue (R-fraction, yield 1.27 g) was extensively washed, dialyzed and freeze-dried. Characterization of this fraction (R-) is the subject of this paper.

Cell wall polysaccharide samples were hydrolyzed in 2M HCl at 100°C for 2 hrs in a sealed ampoule. Hydrochloric acid was removed by evaporation in vacuo at 40°C with repeated additions of water. The hydrolysate was stored over KOH in vacuo to remove residual traces of HCl. Hydrolysates were examined by paper and gas-liquid chromatography. Partial hydrolysis was carried out according to the method of Johnston (16). A 20 mg sample of polysaccharide was suspended in 0.5 ml 88% formic acid, heated at 100°C for 10 min., followed by the

addition of 4 ml 0.4N H_2SO_4 and continued heating for 1 hr. Neutralization was accomplished with $BaCO_3$ and the supernatant concentrated under reduced pressure. The hydrolysate was examined by paper chromatography.

Chromatography on Whatman No. 1 paper was accomplished in the descending manner using (A) ethyl acetate-pyridine-H_2O (12:5:4, v/v) (B) butanol-pyridine-H_2O (2:1:1, v/v). Detection of the reducing sugars was with alkaline $AgNO_3$ (17) and amino sugars were detected with ninhydrin (17). Preparative paper chromatography was carried out on Whatman 3MM paper using the same technique and solvent system A for development. A strip was cut from the center of the chromatogram and the reducing sugars were detected on this strip with alkaline $AgNO_3$. Strips corresponding to the location of the desired sugar were cut from the chromatogram and eluted with water. Sugar alcohols were separated by cellulose F (E. Merck) thin-layer chromatography using n-propanol-ethyl acetate-water (7:1:2, v/v) for development (18). The sugar alcohols were detected with alkaline $AgNO_3$ (18). Sugar and sugar alcohol acetates were separated by thin-layer chromatography using silica gel G (E. Merck) plates developed with 4% methanol in benzene (v/v) and detected by the ferric hydroxamate reaction (19). Methyl ether sugars obtained on hydrolysis of the permethylated polysaccharides were separated on silica gel G (E. Merck) plates as described by Moczar and Moczar (20).

Gas liquid chromatographic separations were carried out on a Bendix 2500 gas chromatograph equipped with two flame ionization detectors using helium as the carrier gas at a flow rate of 40 ml/min.

The butane boronic acid (BBA) ester derivatives of the reduced monosaccharides obtained by HCl hydrolysis were prepared as described by Eisenberg (21). Separation was on a 3% OV-17 (Chromosorb W HP, 80/100 mesh) 6 ft glass column as recommended. The column temperature was 220°C; the inlet and transfer temperatures were 240°C. Acetylated sugar alcohols obtained by Smith degradation were separated on a 3% ECNSS-M (Gas Chrom Q, 100/120 mesh) column. The column temperature was 160°C; the inlet and transfer temperatures were 250°C. The alditol acetates of the O-methyl ether sugars were separated and identified by glc using a Bendix 2500 gas chromatograph and by glc-ms using a Varian 2700 gas chromatograph interfaced to a JEOL-D-100 mass spectrometer with a dedicated TI 980 computer. Separation by glc was accomplished on a 6 ft glass column packed with 3% ECNSS-M on Gas Chrom Q (100/120 mesh). The inlet and transfer tempertures were 250°C. Temperature programming from 140-180°C at 1°/min was used to resolve all the methyl sugars. Positive identification of methyl glucoses was obtained by comparison to authentic samples. Peak areas were measured by electronic integration. The relative concentration of methyl sugars were calculated assuming the molar response factors are equivalent for all derivatives (22). A 6 ft. 3% OV-225 (Chromosorb W-HP, 80/100 mesh) column programmed 165°-230°C at 2° per minute was used for glc-ms. Mass spectra were taken using an ionization potential of 35eV. Identification was made by comparison to retention times and fragmentation patterns of authentic standards.

The cell wall polysaccharides were twice methylated according to the procedure of Hakomori (23) as modified by Sandford and Conrad

(24). Oligosaccharides were methylated once by the same technique. Samples were dried *in vacuo* at 80°C for 4 hrs prior to methylation. Infrared spectra were taken as a thin film evaporated from a $CHCl_3$ solution on sodium chloride plates. Methylated glucan was hydrolyzed by the formic-sulfuric acid method (22). The methyl sugars were identified on thin-layer plates and by gas-liquid chromatography.

Oxidation of the glucan (100 mg) was carried out in 25 mM sodium metaperiodate (100 mls) in the dark at 4°C. The consumption of periodate was determined at various time intervals by the Malaprade method as outlined by Dryhurst (25). Formic acid production was monitored by titration with standard alkali using bromcresol purple as indicator (25). Appropriate blanks were used in each determination. Oxidation was considered complete after 4 days and continued for 5 days. The oxidized glucan was reduced with $NaBH_4$ and hydrolyzed under mild acidic conditions according to the method of Goldstein, *et al.* (26). Glycerol and meso-erythritol were identified by cellulose thin-layer chromatography and as the acetate by gas-liquid chromatography. The 2-O-α-glucopyranosyl erythritol was synthesized according to the procedure of Charlson, *et al.* (27), acetylated with acetic anhydride-pyridine mixture (1:1, v/v) and used as a standard for thin-layer chromatography.

An enzyme mixture containing both β-(1→3) and α-(1→4) glucano-hydrolase activities was recovered from the medium of *S. dimorphosporum* (QM 806) as the 25-50% ammonium sulfate fraction. The precipitate was dissolved in 0.048M citrate-phosphate buffer, pH 4.5, and assayed against various substrates: S-glucan from *S. commune*,

lichenan from Cetraria islandica (Koch-Light), laminarin from Laminaria hyperborea (Koch-Light), pustulan from Parmelia papullosa (Calbiochem), cellulose (Solka-Floc, Brown Co.), carboxymethylcellulose (Eastman Kodak Co.), soluble starch (Fisher), amylose from potato (Sigma), glycogen from shellfish (Sigma), and dextran (Sigma). The enzyme preparation contained both exo-β-(1→3) and α-(1→4) glucanohydrolase activities (Table 1). A liter of culture medium yielded 245 units (28) when assayed against laminarin. Hydrolysis of the isolated polysaccharide and various substrates were carried out in 0.048M citrate-phosphate buffer, pH 4.5, for 12 hrs at 40°C. Toluene was added as a bacterial inhibitor. The amount of enzyme added in each case was 0.06 units per mg substrate. The reaction was stopped by immersion in a boiling water bath for 5 min. Insoluble substrate, if present, was removed by centrifugation prior to the determination of reducing sugar (29) and D-glucose (30). Products of the cell wall R-fraction enzyme hydrolysate were separated by molecular exclusion chromatography using a Bio-gel P2 column (2.5 x 103 cm) with distilled water as the eluant (Fig. 1). Fractions were analysed for total carbohydrate using the anthrone method (31).

RESULTS

The alkali-insoluble fraction, denoted as R-fraction, represents 37% (w/w) of the original lipid-free cell walls. This fraction was extensively hydrolyzed by an enzyme preparation derived from the culture fluid of Sporotrichum dimorphosporum (Table 1). An assay of the enzymic activity indicated significant amounts of β-(1→3) glucanase as expected. However, a thorough examination for various glucanohydrolase activities was carried out and the enzyme preparation also contained a large amount of α-(1→4) glucanase activity (Table 1). A sample (1.0 g) of the R-fraction was treated with the enzyme mixture. The insoluble, enzyme-resistant fraction, designated CORE represented 40% (w/w) of the R-fraction. Soluble products from the enzymic hydrolysate were separated on a Bio-Gel P-2 column and the results are shown in Figure 1. The fractions were combined as indicated and examined by paper chromatography. Fractions I and II were homogeneous and contained reducing material corresponding to glucose (R_G 1.00) and gentiobiose (R_G 0.58) respectively. Fraction III contained three reducing components with R_G's of 0.49, 0.30 and 0.16.

The R-fraction contained primarily glucose and smaller amounts of glucosamine and amino acids as determined by paper chromatography of the HCl hydrolysate. The CORE-fraction also contained mostly glucose and nearly all of the glucosamine of the R-fraction as indicated by the intensities of the spots on paper chromatograms. This fraction also contained amino acids since several ninhydrin-positive components were indicated on paper chromatograms. The HCl hydrolysates of

R-fraction and CORE-fraction were examined by glc, carried out as the butane boronic acid (BBA) ester derivative of the corresponding aldi-tols (21). These results confirmed the presence of glucose and glucosamine. However glc also indicated the occurrence of mannose in small amounts. Quantitation calculated on the basis of relative peak areas of the neutral sugars gave 1.5% and 3.3% mannose in the R- and CORE-fractions respectively.

The R-fraction and CORE were partially hydrolyzed in dilute H_2SO_4 according to the method of Johnston (16). Both fractions gave a similar pattern of reducing sugars on paper chromatograms. In the R-fraction, the disaccharides laminaribiose, maltose, and gentiobiose were identified by comparison to authentic standards using two solvent systems (A & B). Only maltose and gentiobiose were observed in the CORE-fraction. Lichenan, which contains β-(1→3) and β-(1→4) linkages (32) was hydrolyzed under identical conditions and provided a standard for laminaribiose. Trace amounts of glucosamine were present in the partial acid hydrolysates of both fractions.

The R- and CORE- cell wall fractions were twice methylated by the technique of Hakomori (23) as modified by Sandford and Conrad (24). Methylation was judged to be complete since there was an absence of infrared absorption in the region 3400-3500 cm^{-1} in the products. The permethylated cell wall fractions gave on hydrolysis five methyl sugars when examined by silica gel tlc. These were identified as 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, 2,3,6-tri- and 2,3-di-O-methyl-D-glucose by comparison to authentic standards. The identity

of these O-methyl ethers of glucose was confirmed by glc and glc-ms as the alditol acetates (Table 2).

The primary fragments in the mass spectra of the standards were in agreement with the literature (22). Analysis and comparison of the mass spectra confirmed the identification and substitution pattern of the partially methylated alditol acetates. Glc of the mixture also indicated the presence of an additional di-O-methyl ether of glucose, 2,4-di-O-methyl-D-glucose, which apparently was not separated from the 2,3-di-O-methyl-D-glucose by tlc with the solvent system employed. A trace of a mono-O-methyl-D-glucose, identified as the 2-O-methyl ether, was present in the methylated R-fraction hydrolysate, determined by glc. Methylated CORE-fraction hydrolysate contained a significant amount of 2-mono-O-methyl-D-glucose (Table 2).

The cell wall fractions, R- and CORE-, were oxidized with sodium metaperiodate at 22°C in the dark. Moles of periodate consumed and formic acid produced per mole of anhydroglucose (Table 3) were monitored over a period of five days and found to be complete after four days. Methyl-α-D-glucopyranoside was oxidized over the same time period and the results corresponded very well to the expected theoretical values (Table 3). Periodate-oxidized cell wall fractions were reduced to the corresponding polyalcohol and hydrolyzed under mild conditions. After concentration in vacuo at 40°C, and deionization, the polyalcohol hydrolysate was examined by cellulose tlc. Erythritol and glycerol were detected and identified by comparison to authentic standards. These results were also confirmed by silica gel tlc and glc of the corresponding acetate derivatives. Mole ratios of

erythritol to glycerol were determined by glc as the acetates and these results are also given in Table 3. In the acetylated R-fraction polyalcohol hydrolysate, an unknown acetate (R_f 0.23) was indicated on silica gel thin-layer chromatograms. This was subsequently identified as the acetate of 2-O-α-glucopyranosyl erythritol by co-chromatography on tlc plates with a standard prepared according to the method of Charlson, et al. (27). This product was not detected in the CORE fraction.

Oligosaccharides of fraction III from the enzymic hydrolysis were separated by preparative paper chromatography using solvent system A. Each reducing component was eluted with water and the eluate concentrated to dryness in vacuo at 40°C. Component III-3 (R_g 0.49) and III-2 (R_g 0.30) were homogeneous as determined by paper chromatography. The slowest moving oligosaccharide, III-1 (R_g 0.16) contained a trace of component III-2. Fraction II was concentrated by lyophilization and a portion was acetylated using acetic anhydride with sodium acetate as catalyst. The product was recrystallized from ethanol, recovered, dried and had a mp of 191-192°C. The β-octaacetate of gentiobiose has a mp of 193°C (33) which is in agreement with our value and provides unequivocal proof of the identification.

The individual oligosaccharides from fraction III of the enzyme hydrolysate were methylated according to the procedure of Sandford and Conrad (24). Each oligosaccharide was thoroughly dried by storage in a vacuum dessicator over $CaCl_2$ prior to methylation. Silica-gel tlc of the hydrolysates of methylated oligosaccharides III-1 and III-2 gave spots corresponding to 2,3,4,6-tetra- and 2,3,4-tri-O-methyl-D-glucose. Methylated oligosaccharide III-3 gave on hydrolysis

a spot which corresponded to 2,3,4-tri-O-methyl-D-glucose and a spot with a slightly faster mobility (R_{tg} 1.03) than 2,3,4,6-tetra-O-methyl-D-glucose (R_{tg} 1.00). This spot may be 2,3,4,6-tetra-O-methyl-D-mannose. The methyl sugars obtained on hydrolysis of the purified oligosaccharides from fraction III were also examined by glc and glc-ms as the alditol acetates. These results are given in Table 4. Yeast mannan methylated similarly provided a standard for 2,3,4,6-tetra-O-methyl-D-mannose. The mass spectrum of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol and the corresponding glucose analog were nearly identical. Isomers with the same substitution pattern give very similar mass spectra (22). Tlc and glc indicated the absence of di- and mono-O-methyl sugars in any of the oligosaccharide hydrolysates. Based on these methylation results, oligosaccharide III-1 is identified as gentiotetraose and III-2 is identified as gentiotriose. Oligosaccharide III-3 probably contains mannose as an end group, however the mole ratio of the products remains suspect. It is conceivable that one or more constituents of the oligosaccharide were not separated by glc, either remaining on the column or lost by decomposition.

An amino acid analysis was carried out on the R-fraction, CORE-fraction, and the insoluble fraction obtained on treatment of CORE with periodate for five days. These results are shown in Table 5. All of the fractions appear to have a similar amino acid composition, containing high proportions (> 10%, w/w) of aspartic acid, glycine, and leucine. The presence of proline in all of the fractions should be noted. Proline is an amino acid characteristic of many structural proteins.

The glucosamine content of the whole cell wall, the R-, and CORE-fractions were determined by the Elson-Morgan method (34) as modified by Boas (35). Cell wall samples were hydrolysed according to conditions described by Michalenko, et al. (36). These conditions allowed maximum release of glucosamine from the cell walls of Agaricus bisporus. Chitin, purified as described by Barker, et al. (37) was hydrolysed under identical conditions releasing 61.1% glucosamine from chitin on a weight basis. A C,H,N analysis of the purified chitin gave: 45.83% C, 7.10% H, 7.70% N indicating approximately 50% de-N-acetylation. Whole cell walls, the R-fraction, and CORE-fraction of C. macrorhizus microsporus contained 8.6%, 16.0%, and 22.8% chitin respectively.

The insoluble residue recovered after periodate oxidation of the R-fraction and CORE-fraction was washed extensively and freeze-dried. These oxidized samples were hydrolysed and the products examined by glc as the BBA ester derivatives of the alditols. Glucose and glucosamine were identified in both samples by comparison to the retention times of authentic standards. Only a trace of mannose was detected in both oxidized fractions, suggesting that the mannose present in the wall fractions, R- and CORE-, is periodate-sensitive.

Crosses between the monokaryons strain 5377 C. macrorhizus microsporus and the mutant strain 5074 (ATCC 34960), a producer of hydroxylagopodin B (38) were carried out on fruiting medium. In each case, sporulating fruiting bodies were obtained which autolysed to a black amorphous masses after 5-6 hrs., characteristic of the genus Coprinus.

Lagopodin B (39), a sesquiterpenoid hydroxybenzoquinone, was isolated and crystallized from the filtered-spent medium of C. macrorhizus microsporus (5377). Identification was made on the basis of melting point (109-111°C), the violet color of alkaline solutions, the mass spectrum (M^+ 262), and co-chromatography with lagopodin B obtained from C. lagopus (=cinereus) (39). Lagopodin B may be a secondary metabolite typical of the genus Coprinus.

DISCUSSION

A β-(1→3), (1→6)-glucan appears to be a wall component in many species of filamentous fungi and yeasts (40). The polysaccharide is not a linear β-(1→3)-glucose polymer, but contains β-(1→6) branches as demonstrated by methylation analysis and the detection of gentiobiose after enzymic hydrolysis with β-(1→3) glucanases. In most cases, this polysaccharide is found in the alkali-insoluble fraction of the cell walls (40). In the Basidiomycete, Schizophyllum commune, the alkali-insoluble polysaccharide (R-glucan) has been studied in some detail (9,10). It is a highly branched polysaccharide containing β-(1→3) and β-(1→6) linkages in nearly equal amounts. Gentiobiose and higher β-(1→6)-linked oligosaccharides, probably present as branches on a β-(1→3)-linked chain, were characterized as products of enzymic hydrolysis with a purified exo-β-(1→3) glucanase.

Sietsma and Wessels (10) proposed that the insoluble residue remaining after enzymic digestion (a limit polysaccharide) is a mixed-linked glucan, probably associated with chitin, containing both (1→3) and (1→6) linkages with no branch points. The absence of (1→3,1→6)-branch points in the limit polysaccharide and 3-O-β-gentiobiosyl-D-glucose in the hydrolysate, suggests that the exo-β-(1→3) glucanase is capable of hydrolyzing these branch points. These results are at variance with the results of Bush and Horisberger (41) and Nakajima, et al. (42) who found that the β-(1→3,1→6)-branch points are resistant to hydrolysis by the exo-β-(1→3) glucanase. Laminaribiose and 3-O-β-gentiobiosyl-D-glucose were identified as products of exo-β-(1→3) glucanase hydrolysis of the β-glucan from the walls of Piricularia

oryzae (42). However, in a later publication Nakajima, et al. (43) report finding gentiobiose and gentiotriose in the enzymic hydrolysate but not laminaribiose. Bush and Horisberger (41) also found gentiobiose and no laminaribiose in the enzymic hydrolysate of their fungal cell wall polysaccharide. Siehr (9) identified gentiobiose and laminaribiose in the enzymic hydrolysate of S. commune R-glucan. These discrepancies suggest that the exo-β-(1→3) glucanase preparations used for the hydrolysis of fungal cell wall polysaccharides may contain an additional glucanohydrolase activity with a different mode of action. It is also possible that the enzyme may be capable of hydrolyzing the β-(1→3,1→6)-branch points depending on the structure of the substrate.

We have found gentiobiose in the enzymic hydrolysate of C. macrorhizus microsporus R-fraction but no laminaribiose. The fact that the enzyme resistant residue contains no evidence of branching in the residual (1→4)-linked portion but only (1→3,1→6) branch points suggests that the majority of the gentiobiose is derived from the (1→4)-linked portion of the C. macrorhizus microsporus alkali-insoluble polysaccharide. Since we have evidence that our enzyme preparation contains α-(1→4) glucanohydrolase activity, preparations of this enzyme used by other workers may have also contained such activity. It may be that the exo-β-(1→3) glucanase does not cleave the β-(1→3) linkages at branch points. If it does, there may be limitations as to the number of residues in the side chain. Sietsma and Wessels (10) recovered a homologous series of β-(1→6)-linked residues suggesting that branch points containing up to at least six

glucose residues were cleaved by the exo-β-(1→3) glucanase. We have found small amounts of two trimers and a tetramer in addition to a large quantity of gentiobiose released during the hydrolysis of the R-fraction from C. macrorhizus microsporus by the enzyme.

These results suggest that perhaps the true specificity of S. dimorphosporum exo-β-(1→3) glucanase is still not known.

An exocellular polysaccharide mucilage, loosely bound to the cell wall is also produced by most strains of S. commune (44). The structure of this mucilage has been determined as a β-(1→3)-linked glucan with branches of single glucose units attached by β-(1→6) linkages (45). Hydrolysis by the exo-β-(1→3) glucanase gives glucose and gentiobiose (46). The alkali-soluble polysaccharide, S-glucan, located at the outside of the hyphal wall (46), is a straight chain α-(1→3) glucan with α-(1→6) branch points (9). In many cases, including many Basidiomycetes and Ascomycetes (40), the alkali-soluble glucan has been shown to be an α-linked glucan.

An alkali-soluble glucan extracted from the hyphal walls of Coprinus lagopus (=cinereus) has been characterized as β-(1→3) linked glucan with a β-(1→6) link every five internal glucose units (12) distinctly different from that reported in the near relative S. commune. We have reported on the structure of an alkali-soluble (1M NaOH, 18 hrs, 22°C) polysaccharide from the cell walls of Coprinus macrorhizus microsporus (13). The polysaccharide contains primarily α-(1→4), linkages with smaller amounts of β-(1→3), and β-(1→6) linkages. When compared to the alkali-soluble fraction from the generically related C. lagopus, its structure is significantly

different. A diversity in polysaccharide structure is indicated and suggests that the assumption of similar structures for taxonomically-related fungi may not be valid.

The alkali-insoluble (R-) fraction from the cell walls of C. macrorhizus microsporus differs considerably from the R-glucan in S. commune. Methylation of the R-fraction indicates a (1→4)-, (1→3)-linked glucose polymer containing (1→6) branch points (Table 2). The linkages were characterized as α-(1→4), β-(1→3) and β-(1→6) by the identification of maltose, laminaribiose and gentiobiose in the partial acid hydrolysate. Enzymic hydrolysis with an enzyme preparation from Sporotrichum dimorphosporum supported this conclusion (Table 1). Glucose and gentiobiose were the major products (Fig. 1), identified by paper chromatography and silica gel chromatography of the acetylated product. Identification of gentiobiose was substantiated by preparation of the crystalline β-octaacetate. The fraction containing gentiobiose contained no N-acetylglucosamine. Sietsma and Wessels (10) have reported that a similar fraction from S. commune R-glucan contained N-acetylglucosamine. Gentiotriose and gentiotetraose were identified in fraction III eluate from the Bio Gel P-2 column. An oligosaccharide containing mannose as an end group was also present in fraction III. The mannose-containing oligosaccharide is probably a trisaccharide based on its elution position on molecular exclusion chromatography and mobility on paper chromatograms. From the enzymic hydrolysate of S. commune R-glucan, a homologous series of β-(1→6) linked oligosaccharides was obtained (10). The basis for their identity was that a semilogarithmic plot of R_f values against fraction

number gave a straight line. In contrast, we have identified purified portions of the homologous series in C. macrorhizus microsporus cell walls by methylation and analysis of the subsequent hydrolysate. The amount of material in the fractions subsequent to fraction II are small and may not be completely homogeneous as indicated from our findings with fraction III. Preparation of larger samples of these later fractions would be desirable so that they could be unequivocally characterized.

Similar structures for a portion of the R-glucan of S. commune and C. macrorhizus microsporus are indicated by the fact that the products obtained on enzymic hydrolysis are in part identical. However, C. macrorhizus microsporus R-fraction contains α-(1→4) linkages in high proportion (Table 2). These linkages are cleaved by an α-(1→4) glucanohydrolase present in the enzyme preparation from S. dimorphosporum (Table 1). The mode of action of this α-(1→4) glucanase seems to be similar to glucoamylase from Aspergillus, which releases molecules of D-glucose in a stepwise fashion from the non-reducing ends (47). No maltose or higher α-(1→4)-linked oligosaccharides were detected in the R-fraction enzymic hydrolysate.

The R-fraction is a highly-branched glucan and it contains nearly the same mole proportion of linkage-types as the alkali-soluble polysaccharide, however, it is less highly branched and contains more (1→3) linkages as determined by methylation. Nearly equimolar amounts of 2,4-di-O-methyl-D-glucose and 2,3-di-O-methyl-D-glucose were detected and the sum of the di-O-methyl glucoses is nearly equal to the amount of end groups (Table 2), suggesting no crosslinking between

the glucan chains. Identification of 2-O-α-glucopyranosyl erythritol in the hydrolysate of the polyalcohol derived from periodate-oxidation of R-fraction suggests segments of α-(1→4)-linked glucose residues attached through an occasional (1→3) linkage. This was also found to be the case in the alkali-soluble polysaccharide (13).

The CORE-fraction differs considerably from the R-fraction as would be expected (Table 2). In this case 1 in 3 moles of glucose is a non-reducing end group (Table 2). In addition a significant amount of a mono-O-methyl sugar, identified as the 2-mono-O-methyl ether of glucose, was detected. This suggests incomplete methylation. However, this may not be the case since no absorption for OH was detected in the infrared spectrum of the methylated CORE-fraction. The occurrence of 2-mono-O-methyl-D-glucose in the CORE-fraction probably is a structural feature. Further support for this structure is the fact that the amount of end groups equals the sum of the 2,4-di-O-methyl amount plus twice the amount of 2-mono-O-methyl-D-glucose. Also, since no 2-O-α-glucopyranosyl erythritol was detected in the Smith degradation of this fraction, it is logical to conclude that the (1→4)-linked glucan may be attached to the remainder of the polysaccharide through a glucose residue linked at positions O-1, O-3, O-4 and O-6. It is interesting to note that no 2,3-di-O-methyl-D-glucose was detected in the CORE-fraction. Most of the β-(1→3,1→6)-branch points are resistant to hydrolysis by the exo-β-(1→3) glucanase as was found by Bush and Horisberger (41) and Nakajima, et al. (42). Therefore, gentiobiose and higher β-(1→6) linked oligosaccharides identified in the enzyme hydrolysate may be derived primarily from branch points in (1→4)-linked regions.

The results of the periodate oxidation (Table 3) are not totally in agreement with the methylation data. This is explained by the fact that a significant portion of both fractions is chitin and protein. Deviation from expected periodate-oxidation results is much greater for the CORE-fraction since an even larger proportion of the fraction is chitin and protein. Erythritol and glycerol were detected in the hydrolysate of the polyalcohol obtained on reduction of the periodate-oxidized fractions which further supports methylation data indicating the occurrence of (1→4)- and (1→6)- linked residues.

Mannose, which occurs in both fractions in small amounts, was found to be periodate-sensitive. This suggests that the mannose occurs either as end groups, or reducing terminals or linked glycosidically such that it is susceptible to oxidation by periodate. In the alkali-soluble fraction, some of the mannose may be linked differently (13).

Significant structural differences have been noted in the alkali-soluble polysaccharides from C. lagopus (=cinereus) and C. macrorhizus microsporus (13). In addition, the chitin content differs substantially from that of C. cinereus. Whole monokaryotic cell walls of C. macrorhizus microsporus contain 8.6% (w./w.) chitin in contrast to the 26.8-33.4% (w./w.) chitin reported for C. cinereus monokaryotic walls (48). Schizophyllum commune contains 11-13% chitin (10,49) comparable to the content of C. macrorhizus microsporus.

The amino acid content of the various cell wall fractions, including the insoluble residue obtained after periodate oxidation, differed only slightly in each case. Each fraction contained

relatively high amounts of aspartic acid, glycine and leucine. Amino acid analysis of the alkali-soluble polysaccharide gave similar results, but also contained larger amounts of glutamic acid and significantly less proline. In cases where the amino acid content of fungal cell walls has been examined, high proportions of aspartic acid, glutamic acid, alanine, and glycine and the occurrence of proline have been noted (50-53). There seems to be increasing evidence that the protein present in fungal cell wall is an integral component and may be structurally similar in all filamentous fungi.

In conclusion, the cell wall of C. macrorhizus microsporus may be fractionated into two components based on solubility in alkali. Both fractions are similar in structure and contain a mixture of α-(1→4), β-(1→3) and β-(1→6) linkages. Most of the α-(1→4) linked segments are attached through an occasional β-(1→3) glycosidic bond. After enzymic digestion of the R-fraction with a mixture of β-(1→3) and α-(1→4) glucanohydrolase, a CORE-fraction is obtained which on methylation analysis indicates glucose residues which are linked through the O-1, O-3, O-4 and O-6 positions.

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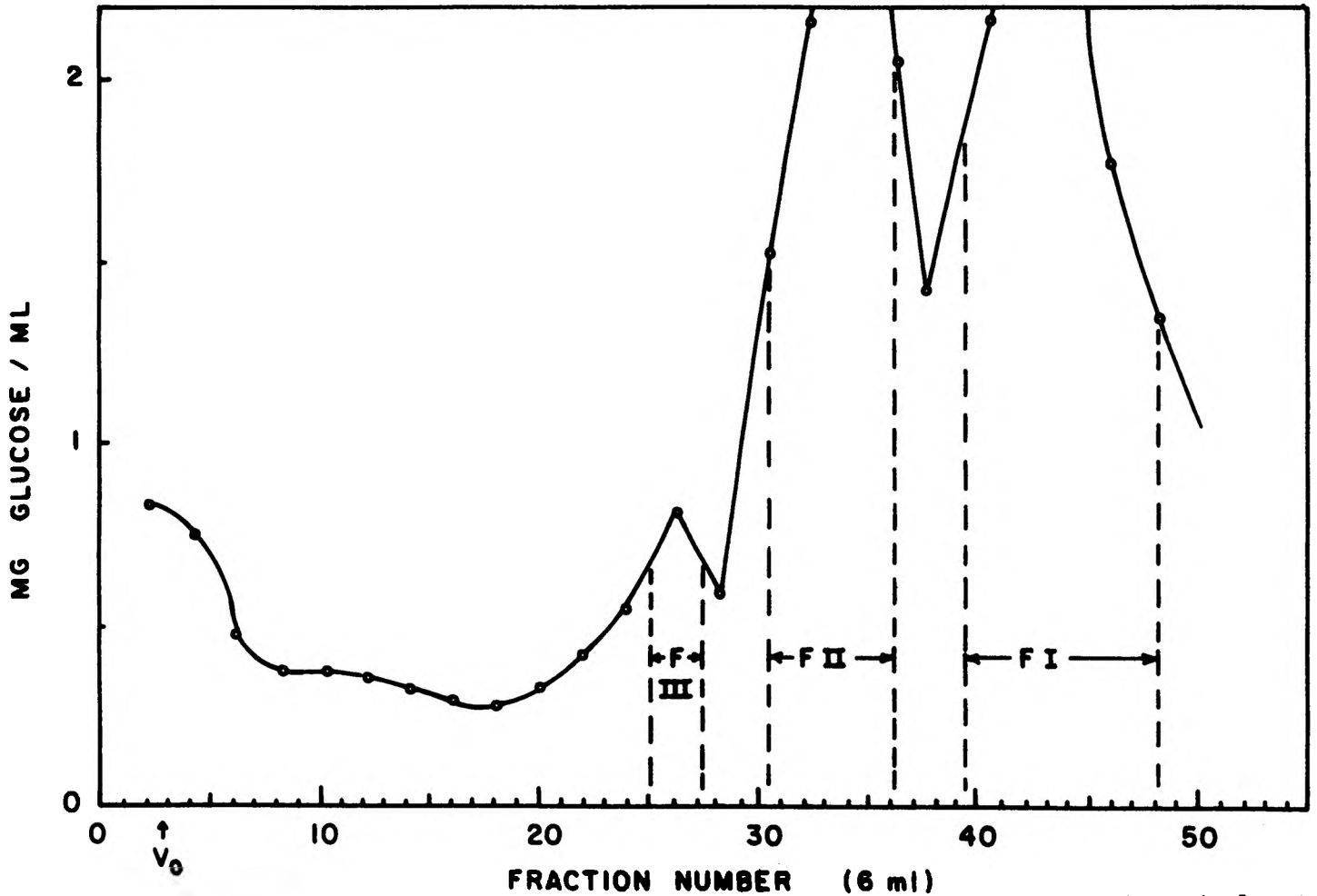

Figure 1. Column Chromatography on Bio-Gel P2 of the R-fraction Enzymic Hydrolysate. V_0, void volume.

TABLE 1. Enzymic hydrolysis of C. macrorhizus microsporus cell wall fractions and other glucans

Substrate Glucan type	Reducing sugar released (%) total by weight	Glucose released (%) total by weight
C. macrorhizus microsporus cell wall fractions		
1. Alkali-soluble fraction	76%	65%
2. Alkali-insoluble (R-) fraction	46%	26%
S-glucan (S. commune) [(1→3-α,(1→6)-α-D-glucan]	4.2%	3.0%
lichenan [(1→3-β,(1→4)-β-D-glucan]	13%	3.7%
laminarin [(1→3)-β-D-glucan]	89%	84%
pustulan [(1→6)-β-D-glucan]	0%	0%
cellulose (Solka-Floc) [(1→4)-β-D-glucan]	0.2%	0.1%
carboxymethylcellulose [(1→4)-β-D-glucan]	0.3%	0.2%
soluble starch [(1→4)-α-,(1→6)-α-D-glucan]	9.0%	9.0%
amylose [(1→4)-α-D-glucan]	53%	57%[a]
glycogen [(1→4)-α-,(1→6)-α-D-glucan]	90%	95%[a]
dextran [(1→6)-α-D-glucan]	11%	11%

[a]Enzyme was not completely inactivated by 5 min at 100°C.

TABLE 2. Methylation analysis of C. macrorhizus microsporus cell wall fractions

O-methyl sugar (alditol acetate)	Mole percentage	
	R-fraction	CORE-fraction
2,3,4,6-tetra-O-methyl-D-glucose	17.2%	31.2%
2,4,6-tri-O-methyl-D-glucose	8.0%	22.3%
2,3,4-tri-O-methyl-D-glucose	7.7%	10.2%
2,3,6-tri-O-methyl-D-glucose	49.4%	12.7%
2,4-di-O-methyl-D-glucose	8.5%	15.3%
2,3-di-O-methyl-D-glucose	9.3%	0%
2-mono-O-methyl-D-glucose	t[a]	8.3%

[a]trace

TABLE 3. Periodate oxidation of C. macrorhizus microsporus cell wall fractions

Substrate	moles IO_4^- consumed[a]/mole anhydroglucose	moles HCOOH produced[b]/mole anhydroglucose	mole ratio[c] erythritol/glycerol
Methyl-α-D-gluco-pyranoside	2.03	0.99	
Alkali-soluble fraction	0.95	0.19	2.1/1
R-fraction	0.94	0.35	0.7/1
CORE-fraction	0.69	0.30	0.2/1

[a]Malaprade method

[b]standard alkali

[c]glc as acetate

TABLE 4. Methylation analysis of Bio-gel P-2 column fraction III oligosaccharides from R-fraction enzyme hydrolysate

O-methyl sugar (alditol acetate)	Mole ratios			RRT[a]
	III-1	III-2	III-3	
2,3,4,6-tetra-O-methyl-D-mannose	--	--	1.8	0.97
2,3,4,6-tetra-O-methyl-D-glucose	1.0	1.0	--	1.00
2,3,4-tri-O-methyl-D-glucose	3.0	1.8	1.0	2.07

Analysed on a 6 ft 3% ECNSS-M (Gas-Chrom Q) glass column. The column temperature was programmed from 140° to 180°C at 1° per minute.

[a]Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol

TABLE 5. Amino acid analysis of *C. macrorhizus microsporus* cell wall fractions

	μmole amino acid/1000 μmole		
Amino acid	R-fraction	CORE-fraction	Periodate-oxidized CORE-fraction
aspartic acid	118	126	129
threonine	56	72	74
serine	72	94	84
glutamic acid	78	72	71
proline	55	54	74
glycine	129	124	117
alanine	77	88	92
valine	57	46	56
isoleucine	61	53	56
leucine	121	106	112
tyrosine	43	50	31
phenylalanine	53	48	50
histidine	16	13	13
lysine	47	38	24
arginine	18	18	17

VITA

Carey Bernard Bottom was born on November 10, 1950, in Boonville, Missouri. He received his primary education at American schools in Bussac, France, Hanau and Berlin, Germany and at Fort Leonard Wood, Missouri. He attended Berlin American High School, Berlin, Germany, in 1964-65 and Waynesville High School, Waynesville, Missouri, during the period 1965-68, graduating in May, 1968. In September, 1968, he enrolled at the University of Missouri-Rolla, Rolla, Missouri and was graduated in July, 1972 with the Bachelor of Science in Chemistry.

He has been enrolled in the Graduate School of the University of Missouri-Rolla since August, 1972 and has held Graduate Teaching and Research Assistantships during this tenure. The Master of Science in Chemistry was received in December, 1975 and he is currently completing requirements for the degree of Doctor of Philosophy in Chemistry.

As a graduate student, he has authored or co-authored several scientific publications in addition to those described in this dissertation.

Bottom, Carey B., Magruder, Gary C., Siehr, Donald J., Grigoropoulos, Sotirios G., and Clarke, William P., (1976), "The Interference of Elemental Sulfur in the Determination of Trace Organics in Drinking Water by the Carbon Adsorption Method", Journal of Environmental Science and Health, A11 (6), 409-415.

Bottom, Carey B., Hanna, Samir B., and Siehr, Donald J., (1978), "Mechanism of the Ninhydrin Reaction", Biochemical Education, 6 (1), 4-5.

The author was married to Christine Lee Mayhan on December 22, 1975, and they have one son, Shawn Carey.

The author is a member of the American Chemical Society, the Mycological Society of America, and the Honor Society of Phi Kappa Phi.

APPENDIX A
STANDARDS FOR CELL WALL ANALYSIS
I. ISOLATION AND ANALYSIS OF YEAST MANNAN

A. INTRODUCTION

Yeast mannan was isolated and purified from brewer's yeast. This was undertaken so that on methylation and subsequent hydrolysis several O-methyl ethers of mannose would be available as standards. The isolation procedure was taken in part from Cifonelli and Smith [1].

B. EXPERIMENTAL

The following describes the isolation, analysis and methylation of yeast mannan.

1. Isolation. Brewer's yeast (466 g) obtained from Anheuser-Busch, St. Louis, was suspended in 400 mls 1mM citrate-phosphate buffer, pH 7.1. The suspension was autoclaved for 2 hours at 124^{o}C, 19 psi and after cooling the cells were removed by centrifugation at 8000 g (15 min). Recovered cells were re-extracted with 250 mls buffer by autoclaving for 2 hours. The extracts were combined and the yeast mannan precipitated by pouring into an equal volume of cold 95% ethanol. The white, flocculent precipitate was collected by centrifugation at 10000 g (20 min). The crude material was redissolved in water and insoluble material removed by centrifugation. This solution was made alkaline with 10% NaOH and Fehling's solution (copper sulfate pentahydrate 3.5%, Rochelle salt 1.7%, sodium hydroxide 5%) added with stirring until an excess was present as indicated by the color of the solution. Most of the supernatant was removed by decantation followed by washing and decantation. The copper-mannan complex was destroyed by dissolution in 6N HCl. This

solution was filtered through 3 layers of Whatman 1 paper and the yeast mannan precipitated again by pouring the filtrate into 2 volumes ethanol. Some of the solution was decanted and the precipitate was collected by centrifugation. The collected precipitate was dissolved in 30 mls H_2O and made acidic with 8 mls glacial acetic acid. Decolorizing carbon (300 mg) was added; the suspension stirred and the charcoal removed by centrifugation and filtration through a pad of Celite on filter paper. The filtrate was poured into three volumes cold 95% ethanol. The precipitate was recovered by centrifugation and washed with two portions of cold ethanol. Recovered yeast mannan was dissolved in water and freeze-dried. Yield: 2.58 g.

2. Hydrolysis. Yeast mannan was hydrolyzed with 2N HCl at 100°C for 3 hours in a sealed ampoule. The hydrolysate was evaporated to dryness in vacuo at 40°C. Most of the HCl was removed by repeated evaporation with H_2O. The hydrolysate was stored in a vacuum dessicator over KOH.

3. Analysis. The yeast mannan hydrolysate was chromatographed on Whatman No. 1 paper developed in a descending manner with ethyl acetate:pyridine:water (12:5:4, v/v). Reducing sugars were visualized with alkaline $AgNO_3$. The hydrolysate was also examined as the alditol acetates by gas-liquid chromatography using 2m x 2mm i.d. glass column packed with 3% ECNSS-M on Gas Chrom Q.

4. Methylation. Yeast mannan was methylated by the technique of Hakomori [2] as modified by Sandford and Conrad [3]. The permethylated mannan was treated with 88% HCOOH and kept at 100°C for 2 hours. Formic acid was removed by vacuum evaporation at 40°C and the dry

residue taken up in 0.25M H_2SO_4. Hydrolysis was carried out for 12 hours at 100°C [4]. The hydrolysate was neutralized with solid $BaCO_3$ and the precipitated $BaSO_4$ was removed by centrifugation. The alditol acetates were prepared as described by Lindberg [4]. Methyl sugars were analyzed by gas-liquid chromatography on a 2m x 2mm i.d. glass column packed with 3% ECNSS-M on Gas-Chrom Q (100/120 mesh) at 205°C. The inlet and transfer temperatures were 240°C. The column was flushed with helium at a flow rate of 30 mls/min. Peak areas were measured by electronic integration.

C. RESULTS AND DISCUSSION

Yeast mannan isolated from brewer's yeast appeared to be a homopolymer of mannose as indicated by paper and gas-liquid chromatography. Mannose was identified by comparison to an authentic standard.

On methylation followed by hydrolysis, five mannose-O-methyl ethers were obtained (Table I). These were identified by comparison of relative retention times [4]. The mole ratios of methylation products (Table I) agree with the results of Stewart, Menderhansen and Ballou [5]. However, these authors carried out their gas-liquid chromatographic analysis as the methyl-α-D-mannosides and trimethylsilyl derivatives.

TABLE I

METHYLATION ANALYSIS OF YEAST MANNAN

O-methyl Sugar (Alditol Acetate)	Observed Mole Ratio	Literature[1] Mole Ratio
2,3,4,6 -tetra-O-methyl-D-mannose	1.00	1.00
3,4,6 -tri-O-methyl-D-mannose	0.75	0.60
2,4,6 -tri-O-methyl-D-mannose	0.46	0.23
2,3,4 -tri-O-methyl-D-mannose	0.04	0.06
3,4 -di-O-methyl-D-mannose	1.01	0.89

[1]Reported by Stewart, et al. [5].

II. CHITIN PURIFICATION

A. INTRODUCTION

Chitin, a homopolymer of N-acetylglucosamine, was purified to provide a standard for the determination of the chitin content of cell walls. The purification was carried out in part according to the procedure of Barker, et al. [6].

B. EXPERIMENTAL

Fifty grams of powdered crayfish chitin [Pfanstiehl Labs, Waukegan, IL] was suspended in 300 mls 2N HCl and stirred continuously for 36 hours at room temperature. Chitin was recovered by centrifugation at 6000 g (10 min). The residue was washed with distilled H_2O by centrifugation. The HCl-treated chitin was then suspended in 300 mls of 5% (w/v) NaOH and autoclaved for 4 hours at 124°C. The cooled suspension was centrifuged at 6000 g (10 min) and the supernatant discarded. The residue was treated again with 5% NaOH (w/v) by autoclaving at 124°C for 2 hours. Chitin was recovered by centrifugation and treated two more times with hot 5% NaOH (w/v) as before. The residue was washed two times with distilled H_2O by centrifugation, suspended in water, dialyzed overnight against running tap water, and finally exhaustively dialyzed against distilled H_2O. Purified chitin (10.3 g) was recovered by lyophilization.

C. RESULTS AND DISCUSSION

The purification process afforded 10.3 g (20.3% yield) pure chitin which was partially de-N-acetylated. The substantial loss in weight was probably due to the removal of contaminating neutral

sugars, especially glucose. Hydrolysates of unpurified crayfish chitin contained a large amount of glucose as shown by paper chromatography. On purification, the hydrolysates contained glucosamine only; no neutral sugars were evident on paper chromatograms. A commercial C,H,N analysis (Galbraith Laboratories) gave the following results: 45.83% C, 7.10% H, 7.70% N, 0.80% ash. These results indicate partial de-N-acetylation (approximately 50% based on N content), as would be expected on treatment with hot alkali [6].

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